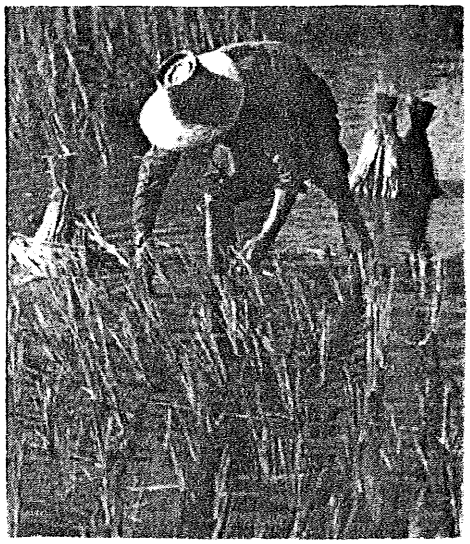
METHANE OXIDATION IN THE RHIZOSPHERE (F300-703)

Model development at the Department of Theoretical Production Ecology
Wageningen Agricultural University

Experiments at the Department of Microbiology
Wageningen Agricultural University



By Liesbeth Mollema (76-07-05-578-090), june 1998

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Voorwoord

Dit verslag beschrijft experimenten en een competitie model met als doel inzicht te krijgen in de invloed van de methanotrofen op de oxidatie van methaan. Dit is belangrijk omdat de emissie van methaan in rijstvelden een grote bijdrage levert aan de totale emissie van dit broeikasgas in de wereld.

De experimenten zijn over het algemeen goed verlopen en daar wil ik dan ook de mensen van de vakgroep Microbiologie onder leiding van Fons Stams voor bedanken. Hierbij wil ik met name Wim Roelofsen bedanken voor zijn snelle ingrijpen bij hapering van de technische apparatuur. Voor het modelleren wil ik met name Peter en Peter bedanken voor hun kritische kanttekeningen. Veel tijd is gaan zitten in het modelleren om het model kloppend te krijgen. Ook was het zeer prettig dat jullie altijd tijd voor me hadden. Peter van Bodegom wil ik met name nog bedanken voor zijn motivering gedurende het onderzoek en zijn bijdrage aan het schrijven van het verslag in het Engels.

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Summary

Rice paddies have a high emission of methane, contributing 20%-30% to global methane emissions. However methane can be oxidised by methanotrophs at the boundary of aerobic/anaerobic interfaces. These interfaces are the rhizosphere and the soil-water interface. The estimation of the percentage of methane oxidised in the rhizosphere is much more variable than at the soil-water interface. For the estimation the most important competitors for the methanotrophs in the rhizosphere have to be taken into account. These are the heterotrophs. Therefore the basis of the mechanistic description is the understanding of the competition between these two bacteria that are both using oxygen. This study describes a competition model for an estimation of the impact of the methanotrophs on the reduction of the methane emission. This model has also been used for a validation of the results of the experiments. These experiments have been done for measuring the growth kinetic parameters of both bacteria. The results of these experiments were not significant, and therefore parameters from literature have been used as input for the model. When describing the experiments with the model the time period for growth in the model was very short. This was due to oxygen limitation. By analysing the model it was possible to explain the low consumption rates in the experiment. It appeared that much parameters are of influence on the consumption rate. At high concentrations the maximum relative growth rate, the amount of biomass and the yield factor had most influence and at low concentrations of the substrate the half saturation constant had more influence. The results of the competition model showed that the methanotrophs could not compete with the heterotrophs in the rhizosphere. This was due to methane limitation and the higher maximum relative growth rate for the heterotrophs. However methanotrophs have also been found in the rice roots. No competition with the heterotrophs takes place in the root, but depending on the concentrations of methane in the roots the influence of the methanotrophs on the oxidation of methane is higher or lower than in the rhizosphere. For a good estimation of the impact of the methanotrophs on the emission of methane the growth kinetic parameters have to be known.

Samenvatting

Rijst velden hebben een hoge methaan emissie, die 20 – 30% bijdraagt aan de methaan emissie in de hele wereld. Methaan kan echter worden geoxideerd door methanotrofen waar aërobe en anaërobe lagen aan elkaar grenzen. Deze grensvlakken zijn de rhizosfeer en de bodem-water overgang. De schatting van het percentage van methaan dat geoxideerd wordt in de rhizosfeer is veel variabeler dan voor de bodem-water overgang. Voor een schatting moeten de meest belangrijke concurrenten voor zuurstof voor de methanotrofen in de rhizosfeer worden bepaald. Dit zijn de heterotrofen. De basis van een mechanistische verklaring is dan ook het begrijpen van de competitie tussen deze twee bacteriën die beide zuurstof gebruiken voor hun groei.
Deze studie beschrijft een competitie model voor het kunnen doen van een schatting van de invloed van de methanotrofen op de reductie van methaan emissie. Dit model is ook gebruikt als validatie voor de resultaten van het experiment. Deze experimenten zijn uitgevoerd om de kinetische parameters te meten voor beide bacteriën. De resultaten van het experiment waren niet significant. Daarom zijn parameters uit de literatuur gebruikt als input voor het model. Bij het beschrijven van de experimenten met het model was de periode voor groei in het model erg kort. Dit werd veroorzaakt door zuurstof limitatie. Bij het analyseren van het model is geprobeerd om de lage consumptie snelheden te verklaren. Het bleek dat veel parameters invloed hebben op de consumptie snelheid. Bij hoge substraat concentraties hebben de maximale relatieve groeisnelheid, de hoeveelheid biomassa en de yield factor het meeste invloed, terwijl bij lage substraat concentraties the half verzadigings constante meer invloed heeft. The resultaten van het competitie model laten zien dat de methanotrofen niet kunnen concurreren met de heterotrofen in de rhizosfeer. Dit kwam door limitatie van methaan en een hogere maximale relatieve groeisnelheid van de heterotrofen. Echter methanotrofen zijn ook gevonden in de wortels van rijst. In de wortel vindt geen competitie plaats met de heterotrofen, maar afhankelijk van de concentratie van methaan in de wortel is de invloed van de methanotrofen op de oxidatie van methaan lager of hoger dan in de rhizosfeer. Voor een goede schatting van de invloed van de methanotrofen op de methaan emissie, moeten de kinetische parameters voor groei bekend zijn.

Chapter 1 Introduction

The oxidation of methane is the subject of this study, which is a part of the project that describes, explains and predicts emissions of methane from rice plants at the level of rice paddies.
Rice paddies have a high emission rate of methane, contributing 20 to 30% to total methane emissions globally. Rice plants enhance methane emission by providing substrates for methanogenesis in forms of root and leaf litter and root exudates. In the research of the project three processes are distinguished: methane production, methane oxidation and transport processes in plant and soil.
Methane oxidation takes place when both oxygen and methane are available. However methane can only be produced in absence of oxygen. Therefore it is expected that oxidation of methane occurs at the boundary of aerobic and anaerobic interfaces. In rice soils these interfaces are the rhizosphere and the soil-water interface. The rhizosphere is the environment near the rice roots, in which the roots produce oxygen which becomes available to the bacteria present in the soil. IN both interfaces oxygen and methane are available via diffusion.

Almost 80% of the methane produced is oxidised at the soil-water interface, by methanotrophs converting methane and oxygen into carbon dioxide and water. Paddy rice grows in water-flooded paddy fields where a thin aerobic soil surface layer and an underlying reduced soil layer develop after submergence. Methane produced in the anaerobic layer must pass through the aerobic surface layer where it can be oxidised by methanotrophs.
For the oxidation of methane in the rhizosphere paddy rice is very important due to its well developed vascular system. This system allows oxygen transport from the foliar parts of the plants to the roots and into the rhizosphere. The presence of oxygen in the rhizosphere both reduces methane production and increases methane oxidation.
The estimation of the percentage of methane oxidised in the rhizosphere is however much more variable. It is difficult to measure methane oxidation in a semi field situation, because methane production always takes place simultaneously. Efforts measuring the methane oxidation by methods of stable isotopes haven't led to results so far. It is still important to describe the methane oxidation in a mechanistic way for an estimation of the impact of the methanotrophs on the reduction of the emission of methane. Therefore it is necessary to understand and quantify the methane oxidation from microbiological processes.

The first step to examine which bacteria are important in the competition for oxygen is to quantify all the bacteria in the rice soil using oxygen. For this aim Most Probable Number countings were used by Boeke (Gerhardt et al., 1981) with different electron donors ($S_2O_3^{2-}$, NH_{4+}, Fe^{2+} and Acetate). It seemed that iron was oxidised chemically with oxygen and that ammonium oxidisers could only grow at 20% oxygen. They didn't grow at 1% oxygen. This means that they are of no importance in a soil with low concentrations of oxygen, like the rhizosphere.
Thiosulfate oxidisers were found in numbers of 10^6 per g soil, while the heterotrophs were found in numbers of 10^9 per g soil. The heterotrophs were the most important competitors of the methanotrophs for oxygen. Therefore the basis of the mechanistic description is to understand the competition between the methanotrophs and the heterotrophs which both use oxygen.

The **objectives** are the following:

1) To quantify the methane oxidation
2) To develop a mechanistic description

Ad 1) The quantification has been done by incubation experiments for pure cultures of the most important oxidisers in the rhizosphere (methanotrophs and heterotrophs). The growth kinetic parameters of both bacteria from the experiment have been compared with parameters found in literature.
Ad 2) A model has been developed for the competition for oxygen between the methanotrophs and the heterotrophs in the rhizosphere.

The **hypotheses** are the following:

1) It was expected that part of the methane that reaches the single root, shall be oxidised by the present methanotrophs.
2) The methanotrophs are not outcompeted by the general heterotrophs

It was necessary to get pure cultures of methanotrophs and heterotrophs. These cultures were used to perform a batch experiment for measuring maximum relative growth rates and half saturation constants for the different substrates (see Chapter 2).
Chapter 3 describes the BOM experiment needed to determine these kinetic parameters. The results of the BOM experiment are used as a comparison with the results of the batch experiment. Chapter 4 compares the results of the model with the results found in the batch experiment for both bacteria. Also a literature review is given in chapter 4 of the kinetic parameters. These parameters will be used as input for the competition model. The aim of this model is to show which bacteria win the competition with the use of the parameters found in literature.
In chapter 5 some conceptual ideas for the extension of the kinetic model to a rhizosphere model are given. In the last chapter the study will be discussed.

Chapter 2 Batch experiments

2.1 Introduction

Batch experiments are needed to measure the kinetic parameters for the growth of methanotrophs and heterotrophs for the quantification of the growth rates of these bacteria and to use the parameters as a input for the model (chapter 4).
Several experiments are available to measure these values. Batch experiments for pure cultures of methanotrophs and of heterotrophs with limitations of different substrates have been chosen. With these experiments the most important kinetic parameters, the half saturation constants for all the substrates and for both bacteria and the maximum relative growth rates are measured in a relatively simple and short way. The results of this experiment have been compared with those found in literature. The results of the model have also been compared with the results of the batch experiments.

2.2 General aspects of the calculation of parameter values

This paragraph describes the calculation of the kinetic parameters of the growth of the methanotrophs and the heterotrophs for the batch experiment.
First, some equations and definitions are given for the growth of the bacteria.
Relative growth rates can be described by a double Monod equation. The double Monod model assumes that the electron donor (methane or acetate) and the electron acceptor (oxygen) combine in the same organism. This assumption is based on the existence of different enzymes that catalyse the respective reductions within the same organism.
Oxygen and methane are needed for growth of the methanotrophs and oxygen and acetate for growth of the heterotrophs.
For the methanotrophs the relative growth rate is described by:

$$\mu = \mu max*([O_2]/([O_2]+KsO_2))*([CH_4]/([CH_4]+KsCH_4))$$

and for the heterotrophs the relative growth rate is described by:

$$\mu = \mu max*([O_2]/([O_2]+KsO_2))*([Ac]/([Ac]+KsAc)).$$

When the substrates are not limiting or when the half saturation constants are much lower than the concentrations at that time, the bacteria will grow at the maximum relative growth rate. The half saturation constant is the concentration of the substrate when the relative growth rate has reached half the value of the maximum relative growth rate.
However the relative growth rate can also be calculated by the maximum consumption rate (Vmax) instead of the maximum relative growth rate. If the maximum consumption rate is used for the calculation of the relative growth rate then the Michaelis Menten constant (Km) is also used instead of the half saturation constant (Ks). Thus the relative growth rate can be calculated by:

$$\mu = ((Vmax*Y)/B)*([O2]/([O2] + KmO2))*([CH4]/([CH4] +KmCH4)).$$

After calculating the relative growth rate, both the yield factor (Y) and the biomass have to been known. The amount of carbon incorporated into the cells per mol carbon in the substrate consumed (yield factor (Y)) is assumed to be constant. The yield factor is however not always constant, for example at lower substrate concentrations due to changes of the maintenance coefficient. The maintenance coefficient is the amount of carbon in the substrate consumed for functions different than for the production of new cell material. However the yield factor and the maintenance coefficient have not been calculated for the experiment but values from literature will be used in chapter 4.
The parameters that have to be measured are μmax, KsO_2, $KsCH_4$ and KsAc.
An assumption that has to be made to calculate the amount of biomass in bacterial carbon per liter is that one bacterium contains $2.07*10^{-13}$ g carbon (Bratbak, 1985; Schlegel, 1972).
The concentrations of the different gaseous substrates were calculated with the universal gas law, P*(V/T)=R*n.
To calculate the concentrations in M in the liquid phase it has to be realised that about 3% of the oxygen and methane dissolves in water at a temperature of 30°C
(Wilhelm et al., 1977).

Some corrections that have to be made are: volume changes by samples taken for measurements of the headspace and of the liquid have to be taken into account, when the concentration of the substrate is converted to the number of moles of a substrate.
For the methanotrophs, samples have been taken from the headspace for the measurement of the concentrations of methane and oxygen. Liquid samples have been taken for the measuring of the optical density and bacteria countings. For the heterotrophs samples have also been taken from the headspace for measurement of the concentration of oxygen. For measurements of acetate, samples have been taken from the liquid phase. This is why more liquid than gas has been taken out from the batches of the heterotrophs.

Calculation of the concentration of the bacteria

The countings of the bacteria by Bürker-Türk have been plotted against the absorption data by optical density measurements (see 2.4, figures 2 and 7).
In case only the optical density has been measured, the amount of bacteria is first corrected for the intersection with the y-axis and then divided by the slope.
The concentration of the bacteria in mol bacterial carbon per liter has been calculated by:
(amount of bacteria / volume of quadrangle in liter)*(g of carbon per bacterium / molmass of carbon).

Calculation of the relative growth rate

The relative growth rates of the bacteria for the different concentrations of the substrates have been calculated by using the formula:

$$((B(T(I+1)) - B(T(I))) / (T(I+1) - T(I)) / ((B(T(I)) + B(T(I+1))) / 2)$$

B = biomass of the bacteria in mol bacterial carbon per liter
T = time in hours
I = number of the time step

The maximum relative growth rate has been calculated by a non-linear optimalisation program (Slide Write) with the data of the relative growth rates and substrate concentrations in time, using the Monod equation.

Calculation of the concentrations of oxygen, methane and acetate

In order to calculate the number of moles of the substrate, first a calibration curve (see Appendix A) has been made. For the calibration of oxygen 0.5 ml of oxygen with a pressure of 1.53 bar (in 1000 ml bottle) has been transferred into a bottle of 120 ml. As a result the pressure in this bottle is 1.15 bar, which has been filled with nitrogen and oxygen. For the calibration of methane, 1.0 ml of methane with a pressure of 1.5 bar (in 1000 ml bottle) and 5 ml of methane with a pressure of 1.5 bar (in 1000 ml bottle) have been transferred into bottles of 120 ml. As a result the pressure in these bottles is 1.25 bar, which has been filled with nitrogen and methane.
In the bottles the concentration of the substrate for calibration has to be measured first. Secondly the original concentration in the batches for the experiment have to be measured. The concentrations above are measured with the following formula:

$$nj = (P1*V1)/R*T;\ norg = (P2*V2)/R*T;\ ntot=(P'*V2)/R*T$$

The actual concentration of the substrate then becomes ntot/V2 multiplied by a dilutionfactor (P1*V1)/(P2*V2):

$$((P1*V1)/(P2*V2)) * P'/(R*T).$$

nj = the number of moles injected
norg = the original number of moles in the batch
ntot = the number of moles injected + the number of moles in the batch
P1 = is the pressure in the 1000 ml bottle in Pa
P2 = is the pressure in the 120 ml bottle with nitrogen in Pa
V1 = is the volume of the sample that is taken to prepare the stock "solution" in m^3
$V2 = 0.12*10^{-3}\ m^3$
P'=((P1*V1) + (P2*V2))/V2.

Thereafter this concentration of the substrate in the 120 ml bottle has been multiplied with the volumes of injected samples for calibration to get the amount of substrate in moles. The number of moles of the substrate have been plotted against the areas of the substrate.
This shows a linear trend with a slope of $2*10^{11}$ area per mol for oxygen and a linear trend with a slope of $2*10^{12}$ area per mol for methane (see Appendix A).
The number of moles in the batches for the experiment has been calculated by dividing the areas by the slope. These amounts have been converted into concentrations in the liquid phase. The number of moles in the batches have been divided first by the volume of the injected sample to get the number of moles in the gas phase. Secondly they have been multiplied by the conversion factor to get the concentration of the substrate in the liquid phase.
The calibration of acetate shows a linear trend, with a slope of $1.28*10^{-4}$ area per M (Appendix A).
The same concentrations of oxygen, methane or acetate are needed in our bottles as the values of the half saturation constants for both bacteria for the different substrates. For example the half saturation constant for oxygen is $2.59*10^{-4}$ M. This is a concentration in the liquid phase. This has been converted to $2.25*10^{-3}$ M in the gas phase for 25 ml of liquid and 95 ml of headspace. The other concentrations that had to be added are five times higher or five times lower. The concentration of oxygen in the other six bottles is not limiting. The number of ml that had to be added to get these concentrations have been calculated. In that case the number of moles in the erlenmeyer of 1000 ml with oxygen or methane has to be known.

Perl = $1.5*10^{5}$ Pa
Verl = $1*10^{-3}$ m^3
R = 8.3145 m^3/(K*mol)
T=303 K

The number of moles is $5.95*10^{-2}$ mol by using the gas law. The concentration of the substrate in the erlenmeyer is $5.95*10^{-2}$ M. For example the number of ml oxygen for the methanotrophs that had to be added is $95/(5.95*10^{-2}/2.25*10^{-3}) = 3.6$ ml.

Calculation of the consumption rates and half saturation constants

The consumption rates above have been measured with the following formula:

$$V = (([S](T(I+1))) - ([S](T(I)))) / ((T(I+1)) - (T(I)))$$

V = the consumption rate in mol substrate per liter per hour
S = the concentration of the substrate in mol substrate per liter
T = time in hours
I = the number of the time step

The half saturation constant has been fitted with a non-linear optimalisation program (Slide Write) with data of the relative growth rates and substrate concentrations, using the Monod equation.

2.3 Material and methods

To measure the parameters for growth like the half saturation constant and the maximum relative growth rate, growth has to be limiting by just one substrate. For the batch experiments 12 bottles of 120 ml have been used for as well the methanotrophs as for the heterotrophs. In 6 bottles oxygen is limiting and in the other 6 bottles acetate or methane is limiting depending on the type of bacterium. The number of bottles has been chosen with the idea to take three different concentrations of the limiting substrate, one concentration the same as the half saturation constant for that substrate estimated from literature and the other two concentrations 5 times lower or higher than the half saturation constant for that substrate. The experiment has been carried out in duplicate. The ratio of headspace/liquid volume in the batch has been taken almost 4, so that the volume of the headspace is large enough for the gases to dissolve in the liquid phase. Besides the bottles have also been kept in shaker at a constant temperature of 30 °C.
Both bacteria are grown in a NMS (Nitrate Mineral Solution) medium containing the following substrates: $MgSO_4$ (1.0 g), $CaCl_2$ (0.2 g), $FeCl_2$ ($4*10^{-3}$ g) and KNO_3 (1.0 g), trace-elements (0.5 ml). Demi water is added to a volume of 1000 ml (by Whittenbury et al., 1970).
The bottles have been filled with medium and pure nitrogen with a pressure of 1.4 bar. The atmosphere has been obtained by repeatedly alternating evacuation of the headspace by a vacuumpump and filling by N_2-gas for 7 times. Thereafter the bottles have been sterilised. After sterilisation oxygen, phosphate buffer and potassiumacetate solution, respectively methane have been added to the bottles for the heterotrophs and the methanotrophs. The different concentrations of gases have been added with a syringe from 1000 ml bottles with 100% oxygen or 100% methane both with a pressure of 1.5 bar. The gases added to the bottles for the experiment have been corrected for the pressure in the bottle with 1000 ml. The different dilutions of potassiumacetate-solution have been made from a 50 mM stock solution and have been added through special bacterial membranes. At the end 1 ml from a fully grown culture of methanotrophs or a fully grown culture of heterotrophs has been added to each bottle.
The general set up of the batch experiments is given in figure 1:

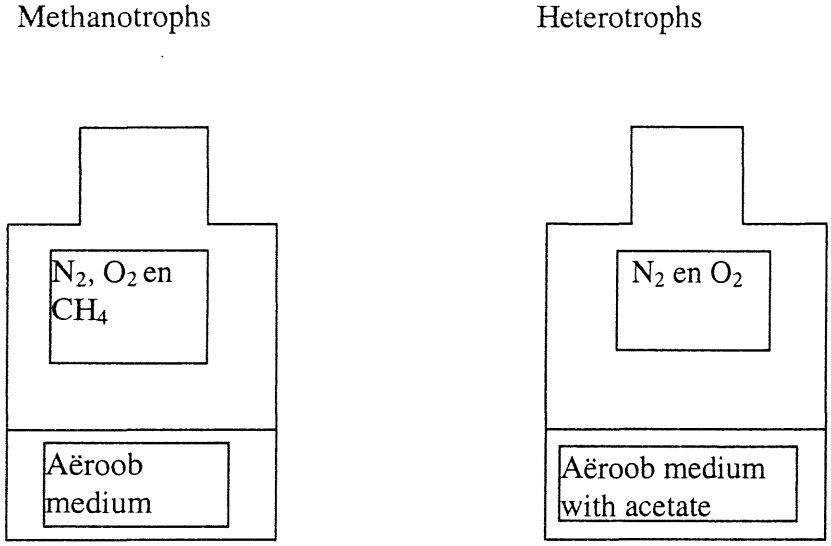


Figure 1: General set up of the batch experiments

The amount of bacteria has been measured in time to be able to calculate the relative growth rates of the bacteria at different concentrations of the substrates. These measurements have been done for the performance of a calibration curve in two ways:

I) with optical density measurements with a photo-spectrometer. Measurements were carried out at a wavelength of 660 nm

II) bacterial countings with the Bürker-Türk counting-chamber.

For the first method 0.5 ml of liquid, taken out of each bottle and 0.5 ml of NMS medium have been added to a cuvet. For the second method the amount of bacteria has been counted in a volume of $2.5*10^{-8}$ ml under a microscope. The volume of one quadrangle is $2.5*10^{-8}$ ml. In total five to ten quadrangles have been counted and the average has been taken.

The concentrations of the substrates have been also measured in time to be able to calculate the consumption rates of the different substrates for both bacteria. The methane concentrations have been measured with a gas chromatograph with a Flame Ionisation Detector (FID). The areas obtained from the samples are related to the calibration curve.

For the determination of acetate concentrations, samples of 0.5 ml of liquid from each bottle have been taken. These samples have been centrifuged for ten minutes. The supernatant has been kept in small vials in a freezer at -20°C until the end of the experiment. All samples have been analysed on a HPLC after 1:1 dilution with 1M formic acid.

Measurements for methanotrophs

The measurements start with the methanotrophs because they grow slowly, therefore they have to be monitored for a longer period. Both oxygen and methane are needed for the growth of the methanotrophs. 12 bottles for the methanotrophs were used. Each bottle contains 25 ml of NMS medium, 0.05 ml of phosphate solution and 95 ml of headspace (oxygen, methane and nitrogen). The concentrations of the oxygen and methane in the liquid phase measured at time zero are presented in tables 1 and 2:

Table 1: Concentrations of oxygen and methane in the liquid phase with a methane limitation

Number of the batch	1	2	3	4	5	6
Concentration of O_2 in M	$6.06*10^{-6}$	$8.64*10^{-6}$	$7.58*10^{-6}$	$5.03*10^{-6}$	$4.68*10^{-6}$	$6*10^{-6}$
Concentration of CH_4 in M	$1.16*10^{-3}$	$2.13*10^{-3}$	$3.12*10^{-5}$	$3.38*10^{-4}$	$5.07*10^{-5}$	$4.82*10^{-4}$

Table 2: Concentrations of oxygen and methane in the liquid phase with a oxygen limitation

Number of the batch	7	8	9	10	11	12
Concentration of O_2 in M	$5.04*10^{-6}$	$5.3*10^{-6}$	$5.65*10^{-6}$	$3.89*10^{-6}$	$4.73*10^{-6}$	$4.45*10^{-6}$
Concentration of CH_4 in M	$1.20*10^{-3}$	$2.03*10^{-3}$	$2.51*10^{-3}$	$2.15*10^{-3}$	$2.25*10^{-3}$	$1.99*10^{-3}$

It appears that the initial concentrations of oxygen are almost the same for the situations with and

without oxygen limitation. This has consequences for the results of this experiment, as the relative growth rate for the methanotrophs with methane limitation would be lower than with oxygen limitation.
Measurements for methane and oxygen have been done at t=0, 24, 48, 72, 143, 191, 383 and 479 hours. The optical density measurements and measurements of the concentrations of oxygen and methane have been done for each sampling moment.
The counting of the bacteria by Bürker-Türk has only been done at t=0 for stock-solution, at t=24 for all the bottles, at t=48 for bottles 1 to 6 and at t=143 it was done for bottle 1, because only in this bottle growth has been occurred, as has been measured by the optical density method.

Measurements for heterotrophs

Both oxygen and acetate are needed for the growth of the heterotrophs.
Also 12 bottles have been used for the heterotrophs. Each bottle contains 25 ml of NMS medium, 0.05 ml of phosphate solution, 1 ml of potassium acetate solution and 94 ml of headspace (oxygen and nitrogen).
The concentrations of the oxygen and acetate at time zero are presented in tables 3 and 4:

Table 3: Concentrations of oxygen and acetate in the liquid phase with a oxygen limitation

Number of the batch	1	2	3	4	5	6
Concentration of O_2 in M	$4.88*10^{-6}$	$4.76*10^{-6}$	$1.02*10^{-6}$	$9.11*10^{-7}$	$2.88*10^{-6}$	$2.05*10^{-6}$
Concentration of Ac in M	$5.57*10^{-3}$	$7.66*10^{-3}$	$5.46*10^{-3}$	$5.16*10^{-3}$	$5.74*10^{-3}$	$5.69*10^{-3}$

Table 4: Concentrations of oxygen and acetate in the liquid phase with a acetate limitation

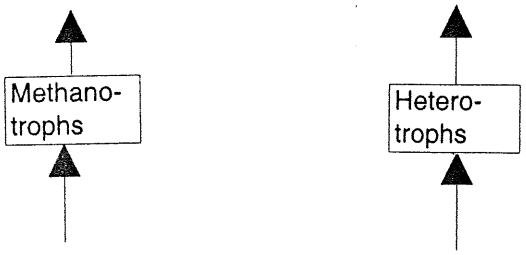
Number of the batch	7	8	9	10	11	12
Concentration of O_2 in M	$1.32*10^{-5}$	$1.21*10^{-5}$	$1.14*10^{-5}$	$1.21*10^{-5}$	$1.09*10^{-5}$	$1.33*10^{-5}$
Concentration of Ac in M	$3.67*10^{-3}$	$2.80*10^{-3}$	$1.37*10^{-3}$	$1.22*10^{-3}$	$6.98*10^{-4}$	6.93810^{-4}

The measurements have been done at t=0, 32, 50, 76, 96, 119 and 150 hours.
The counting of the bacteria has only been done at t=0 and t=119 for all the bottles and at t=50 for the bottles 1 to 5.
The optical density measurements and measurements of the concentrations of potassium acetate and oxygen have been done for each sampling moment.

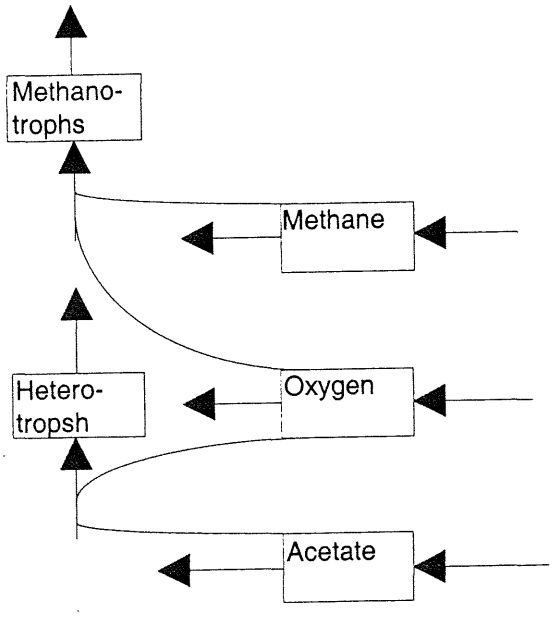
2.4 Results of the batch experiment

To determine the growth of the bacteria the values of the parameters for growth of the bacteria have to be known. The most important kinetic parameters are the half saturation constants for oxygen and carbon substrates and the maximum relative growth rates. An experiment has been done with two types of bacteria with different substrate limitations to define these Monod parameters.
There are four paragraphs and some subparagraphs for the distinction between the results of methanotrophs and the results of heterotrophs. Because the growth of the methanotrophs depends on other substrates than the growth of the heterotrophs a distinction has also been made between different substrate limitations.
The structure of the paragraphs is the following:

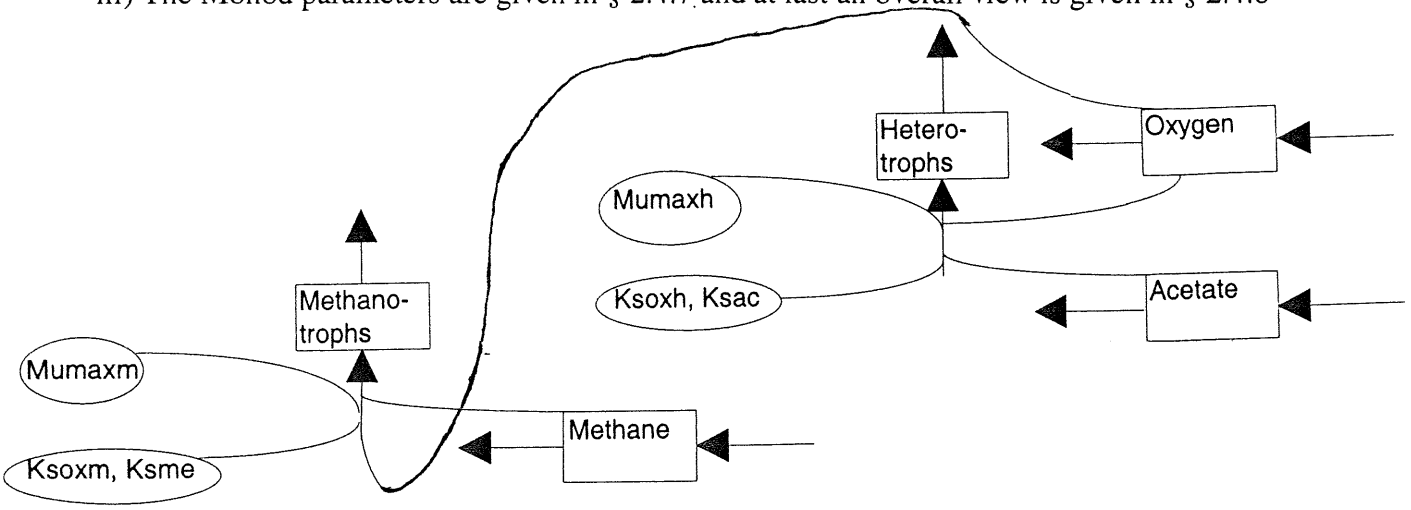
i) The measurement of the biomass and the growth of both bacteria are presented in § 2.4.1 and § 2.4.2



ii) The concentrations and the consumption rates of the substrates are presented in § 2.4.3 – § 2.4.6



iii) The Monod parameters are given in § 2.4.7 and at last an overall view is given in § 2.4.8



Paragraph § 2.4.9 presents the conclusion of the experiment results.
The numbers and symbols in the figures are corresponding to the numbers of the batches. For the methanotrophs in the batches 1 - 6 the concentration of oxygen is non-limiting and the concentration of methane is limiting and in the batches 7 - 12 the concentration of oxygen is limiting and the concentration of methane is non-limiting. For the heterotrophs in the batches 1 - 6 the concentration of oxygen is limiting and the concentration of acetate is non-limiting and in the batches 7 - 12 the concentration of oxygen is non-limiting and the concentration of acetate is limiting. The batches contain different concentrations of the limiting substrate and a similar concentration of the non-limiting substrate (as was shown in tables 1-4).

2.4.1 Biomass of the methanotrophs

The concentration of bacterial biomass has been measured in two ways:
1) by measuring the optical density at 660 nm
2) by counting the number of bacteria with a Bürker-Türk counting-chamber.
Both measurements resulted in densities and have been compared with each other. A linear relationship has been found between the absorption measurements and the counted number of bacteria. The fitted line does not reach the point (0,0), as the absorption has been measured with a standard solution of demi-water which was set at zero, instead of a blank containing NMS medium. If a standard with medium without bacteria would have been chosen, this would also have an absorption. The difference between the standards with demi-water or with medium, both without bacteria, is 0.009 as has been tested by van Bodegom. Due to this difference it was not allowed to fit a line through (0,0). Much data has been found for low numbers of methanotrophs and less for high numbers of methanotrophs, but enough to fit the data. The measurements with the Bürker-Türk counting chamber have been done at the start of the experiment with the idea that the bacteria did not decrease in that time. The fitted line has a significance at $P<0.01$ and thus the line is significant.

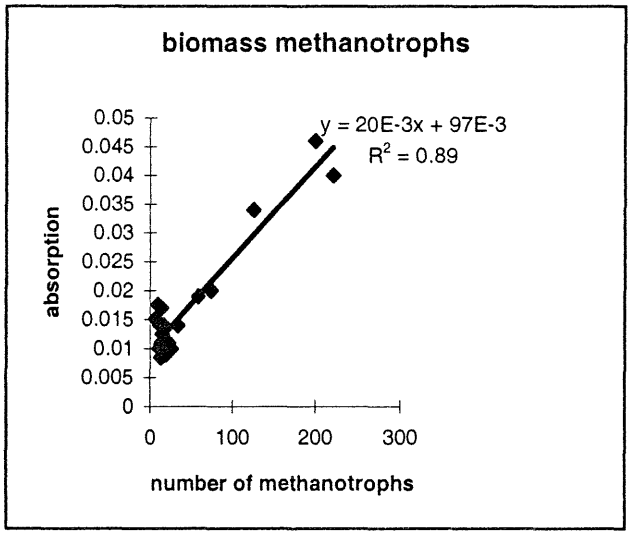


Figure 2: The absorption measurements plotted against the counted number of bacteria.

Figure 2 has been used to derive the numbers of bacteria from optical density measurements. The amount of the methanotrophs has been plotted against time and increased with methane (figure 3). The concentration of the methanotrophs in figure 2 increases very fast in batch 1. This is due to the high concentration of methane found in this batch.
Some values of the concentration of the biomass of the methanotrophs that have been found in literature are $0.129*10^{-3}$ mol/liter in paddy soil in fresh state (Bender et al., 1992), $0.708*10^{-3}$ mol/liter in paddy soil after preincubation under 20% methane (Bender et al., 1992) and $7.39*10^{-5}$ mol/liter in flooded rice soil (Bosse et al., 1997). The concentrations in the experiment are much higher.

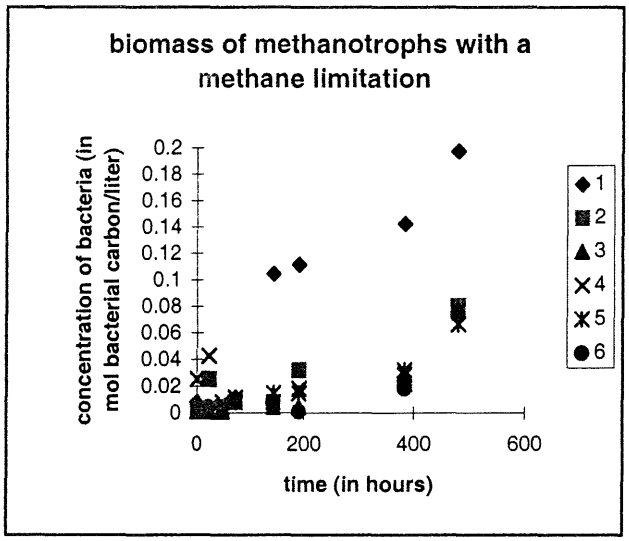


Figure 3: Amount of methanotrophs in mol bacterial carbon per liter with a methane limitation for the batches 1-6

Figure 4 shows that the concentration of methanotrophs also increases in the case that oxygen was the limiting substrate.
It seems that if methane is the limiting substrate the amount of methanotrophs is lower than when oxygen is the limiting substrate, if you don't consider batch 1. Therefore a comparison has to be made between the ratio of the concentration and the half-saturation constant for oxygen and the ratio of the concentration and the half saturation constant for methane when the substrates are limiting. When the concentrations are high of the limiting substrate in comparison to the half-saturation constant of the limiting substrate then the number of biomass can grow with a maximum relative growth rate. Otherwise the relative growth rate will be lower than the maximum relative growth rate and the biomass will also become lower. Another explanation is the low initial values for oxygen that have been measured in the batches with methane limitation (§2.3). It appears that in these batches both substrates are limiting, so lower values of the biomass are found.
There is not much difference in growth between the batches, thus the differences in the relative growth rate for the different concentrations of the substrates are small.

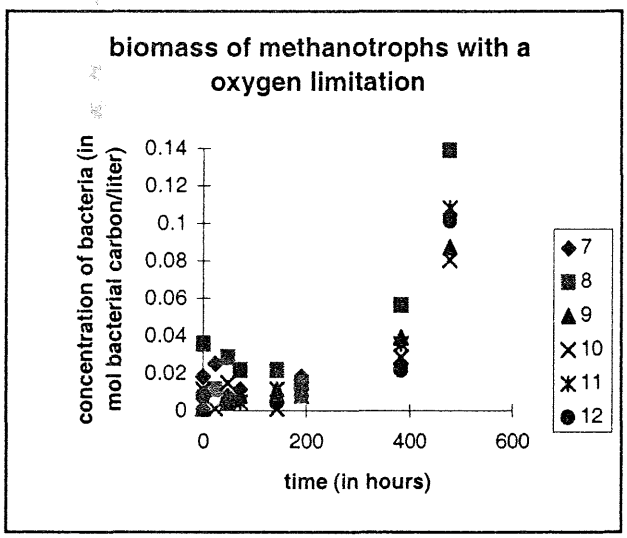


Figure 4: Amount of methanotrophs in mol bacterial carbon per liter with a oxygen limitation for the batches 7-12

The growth rate has been derived from the differentiation of the biomass to time. By dividing the growth rate by the biomass at that time the relative growth rate has been calculated. The relative growth rate decreases no so fast at the start of the experiment, due to the absence of growth at that time. However growth of the biomass with methane limitation is expected at the start of the experiment due to high methane concentrations, but there is hardly any growth of the biomass during the first 8 days. An explanation for the absence of growth could be a low activity of the methanotrophs at the start of the experiment. This has been called a lag-phase after which growth occurs. Low specific activities (Qmax in mol substrate per mol bacterial carbon per hour) have been calculated, especially for oxygen of both bacteria. However these calculated specific activities could be due to high biomass estimations. At the end of the experiment negative relative growth rates were found more constantly, due to decrease of the biomass by decay or mortality.

At the start of the experiment negative relative growth rates have also been found, maybe due to the absence of growth during the first 50 hours. During that time period bacteria could have decreased due to bad circumstances or worse intrinsic characters.
Not much differences in the relative growth rates are found between the batches as is due to the small differences in the biomass.

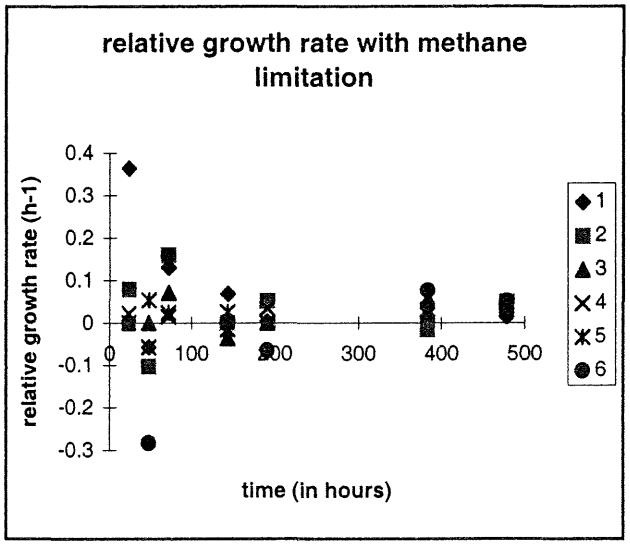


Figure 5: Relative growth rate per hour for the methanotrophs with methane limitation

There is not very much difference between the relative growth rates with a methane limitation or a oxygen limitation (see figures 5 and 6). So comparatively the same limitations are induced to both batch types. This is not the case for the biomass (see figures 3 and 4). However both plots show much scatter of the data that has to be taken into account when interpreting the results.

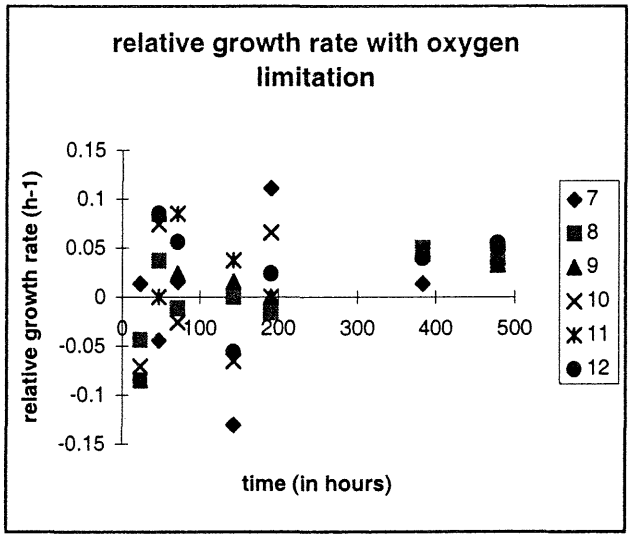


Figure 6: Relative growth rate per hour for the methanotrophs with oxygen limitation

2.4.2 Biomass of the heterotrophs

This paragraph describes the same kind of data for the heterotrophs as has been done for the methanotrophs. First the bacterial biomass has been presented. A linear relationship for the heterotrophs has been found between the two techniques to determine the bacterial biomass. The fitted line does not reach the point (0.0) as for the same reason as in figure 2. The fitted line has a significance at $P<0.01$.
The linear coefficient in figure 7 is a little higher than in figure 2, indicating that the morphological structures of these bacteria are different. For the same absorption value for both bacteria we find a higher number of bacteria for the methanotrophs. Maybe the heterotrophs are more tight together and have been counted as they were just one bacterium, or that the size of the methanotrophs is smaller and therefore they transmit more light.

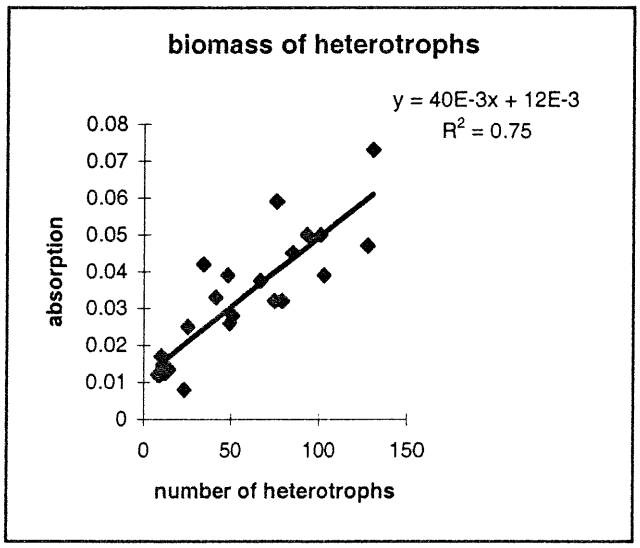


Figure 7: The absorption measurements plotted against the counted numbers of heterotrophs

The next step is to calculate the bacterial biomass in the batches in time. If the biomass of the heterotrophs with a oxygen limitation (figure 8) has been compared to the biomass of the methanotrophs with a oxygen or methane limitation (figures 3 and 4) lower amounts of bacteria have been found for the heterotrophs. An explanation for the lower concentrations of biomass of the heterotrophs can be the lower initial concentrations of oxygen for the heterotrophs. Maybe oxygen or acetate are more limiting for the heterotrophs than oxygen and methane are for the methanotrophs. Figure 10 and 11 show that the relative growth rates of the heterotrophs with oxygen and acetate limitations are lower than for the methanotrophs with methane and oxygen limitations. The lower relative growth rates are due to the lower biomass estimations.
Some concentrations of the biomass of the heterotrophs for pure cultures found in the literature are $17.1*10^{-3}$ - $34.2*10^{-3}$ (Bodelier et al., 1997) and $0.483*10^{-3}$ - $4.17*10^{-3}$ (Gerritse et al., 1992). The amount of the biomass in the experiment is higher.

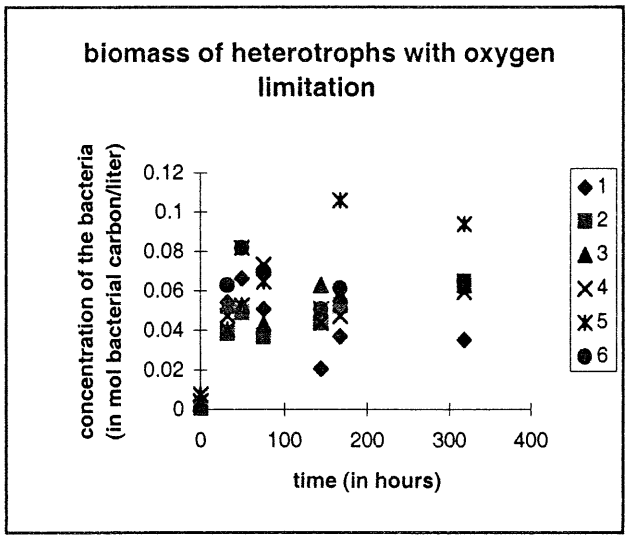


Figure 8: The amount of heterotrophs in mol bacterial carbon per liter with oxygen limitation for the batches 1-6

In the case of acetate limitation, there is also a lot of scatter in the bacterial biomass.

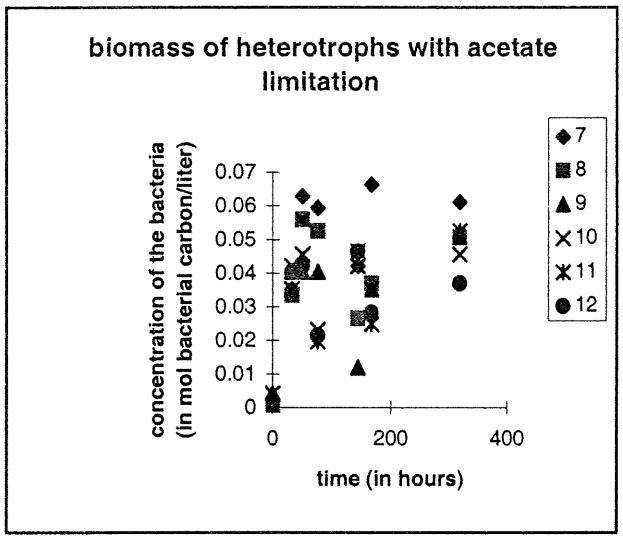


Figure 9: The amount of heterotrophs in mol bacterial carbon per liter with acetate limitation for the batches 7-12

The final step is to calculate the relative growth rates for the two limitations.
First a fast decrease can be seen in the relative growth rate and after 100 hours the amount of heterotrophs decreases in all the batches except in batch 5. This indicates that the oxygen concentration is not enough anymore for growth of the heterotrophs and the bacteria stop growing and decay starts. The relative growth rates for oxygen limitation (figure 10) and for acetate limitation (figure 11) are somewhat lower than found for methanotrophs (figures 6 and 7), because of some different intrinsic characters of the bacteria. The scatter in the relative growth

rates is due to the scattering in the measured bacterial biomass for the heterotrophs.

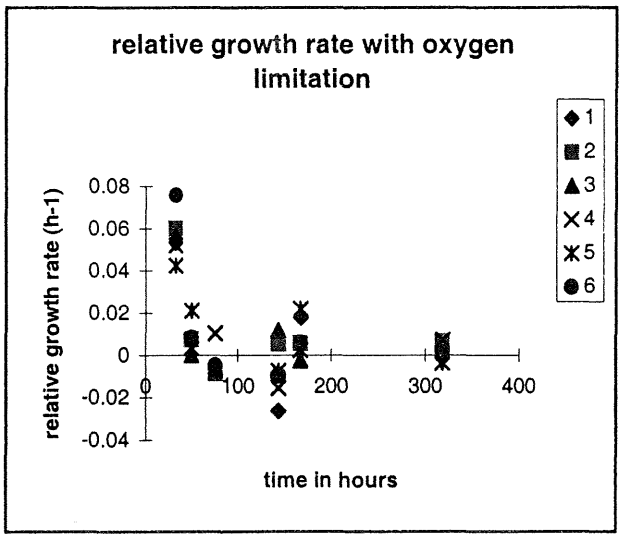


Figure 10: Relative growth rate per hour for the heterotrophs with oxygen limitation

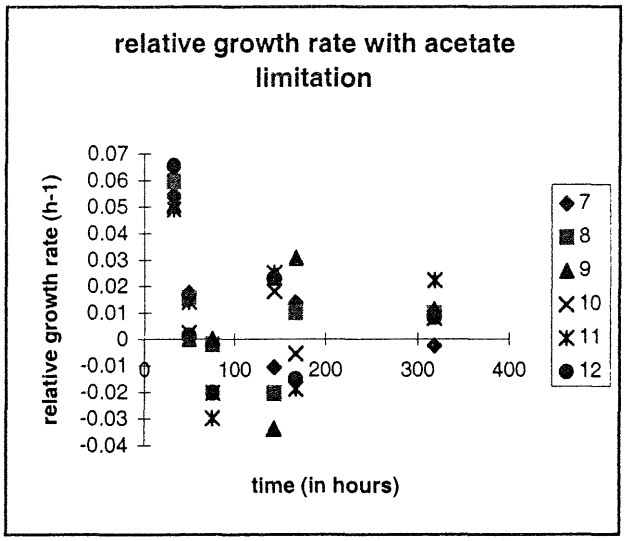


Figure 11: Relative growth rates per hour for the heterotrophs with acetate limitation

The concentration of the biomass and the relative growth rates are higher for the methanotrophs than for the heterotrophs, due to better intrinsic characters of higher concentrations of oxygen. Compared to literature it can be said that the concentrations of the biomass of both bacteria measured in the experiment are higher.

2.4.3 Methane concentrations and consumption rates of methane for the methanotrophs with methane limitation

Paragraph 2.4.3 – 2.4.6 present the measurements of the concentrations and the consumption rates of the substrates.
First the methane concentration for the methanotrophs has been described. Methane is consumed very fast. After 100 hours the methane concentration left in the batches was about $8*10^{-6}$ M, except for batches 1 and 3 which was lower. In these batches still some growth has been found (see figure 3). The half saturation constant for methane should be very low for the methanotrophs to use those low concentrations for growth. Those low values for the half saturation constant of methane have been found in literature for rice soil (see § 4.3). Another explanation could be that the detection limit of the gas chromatograph for the measurements of the concentration of methane is too high. However the detection limit that has been found is $4*10^{-9}$ M. This is within the range of the concentrations of methane measured and which is also far below the Ks values for methane in the literature.

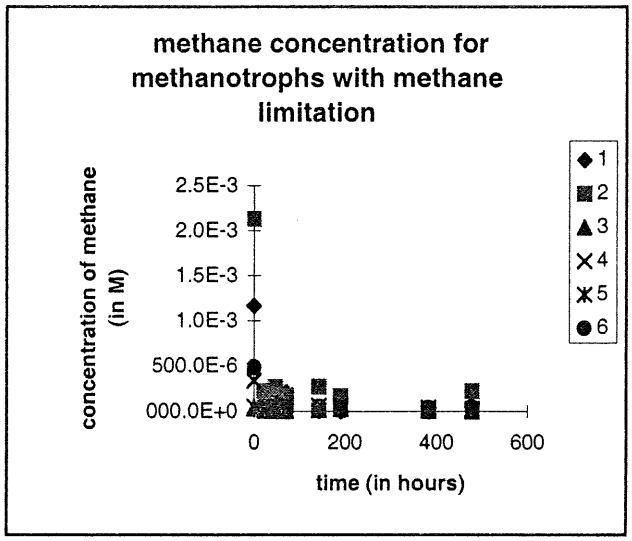


Figure 12: Concentration of methane in mol per liter for methanotrophs with methane

Secondly the consumption rates with methane limitation for the methanotrophs have been calculated. The consumption rates were negative values because the concentrations of oxygen decrease in time. Here the consumption rates were plotted as positive values. Almost all methane is consumed in the first 100 hours. The consumption rates are high at the start of the experiment due to the high concentrations of the substrate at that time. In literature a maximum consumption rate for methane has been found of 1.2*10-6 M/h. In the experiment the consumption rates at t=0 are much higher than in literature (Bosse et al., 1997). However the biomass is also much higher in the experiment than was found by Bosse.

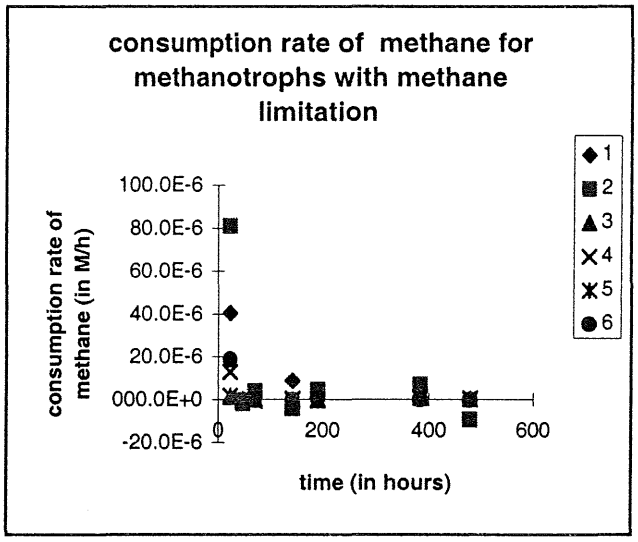


Figure 13: Consumption rate of limited methane in mol per liter per hour for methanotrophs

2.4.4 Oxygen concentrations and consumption rates of oxygen for the methanotrophs with oxygen limitation.

This paragraph describes the concentrations and consumption rates of oxygen for methanotrophs in time. The concentrations decrease in time due to oxygen consumption. In batch 7 and 8 the concentrations of oxygen are higher than in the other batches. After 100 hours the oxygen left in the batches 9 to 12 is very low. However in these batches still growth has been found (see figure 4). This could mean that the half-saturation constant for oxygen has to be very low for the methanotrophs to use those low concentrations of oxygen for growth. However those low half saturation constants have not been found in the literature (see § 4.3). Another explanation could be that the detection limit of the gas chromatograph for measurements of the concentrations of oxygen is too high. The lowest concentration that could- be measured is $2.2*10^{-8}$ M, which was within the range of the concentrations of oxygen that have been measured, and which is also far below the Ks values for oxygen in the literature.

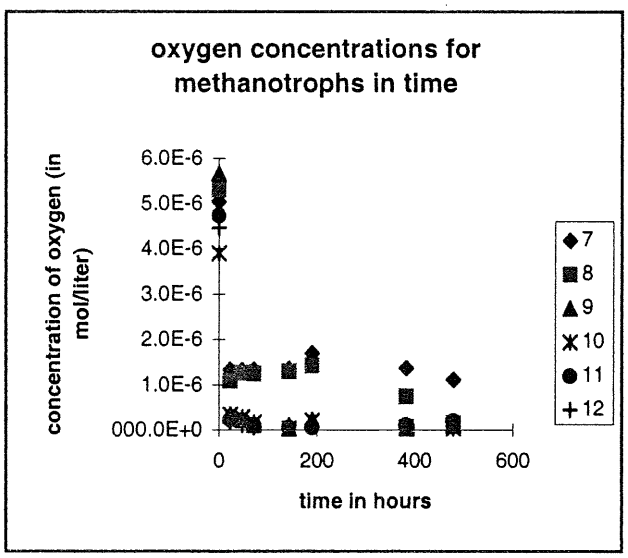


Figure 14: Concentration of oxygen in mol per liter for methanotrophs with oxygen limitation

Secondly the consumption rates for limiting oxygen for the methanotrophs have been calculated. At the start of the experiment the consumption rates are high for the methanotrophs with oxygen limitation because of high concentrations of oxygen. After 100 hours the consumption rate has almost become zero, as almost all oxygen has been consumed. However still growth has been found in the batches with oxygen limitation after 100 hours. Another explanation for this could be that the bacteria decrease their maintenance and therefore more substrate could be consumed for growth.

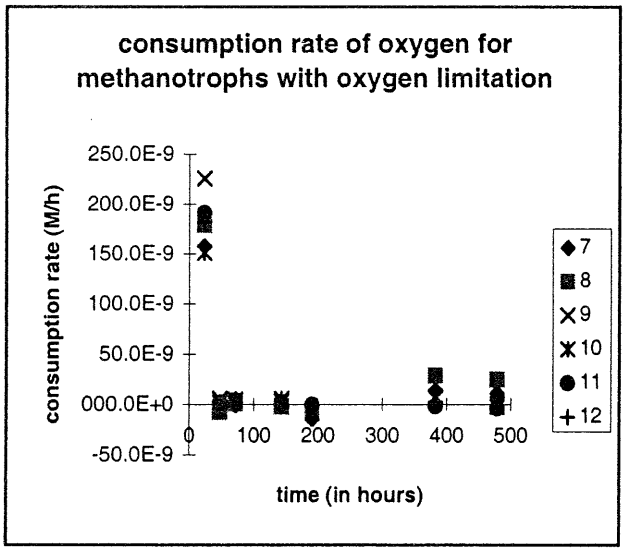


Figure 15: Consumption rate of limited oxygen in mol per liter per hour for the methanotrophs

2.4.5 Oxygen concentrations and consumption rates for the heterotrophs with oxygen limitation

This paragraph describes the oxygen concentrations and the oxygen consumption rates in time for the heterotrophs. The concentration of oxygen decreases fast at the start of the experiment (see figure 16). After 100 hours the oxygen concentration left in the other batches is about $9*10^{-8}$ M. Only in batch 5 some more oxygen has been found after 100 hours. However still some growth has been found in some of these batches after 100 hours.

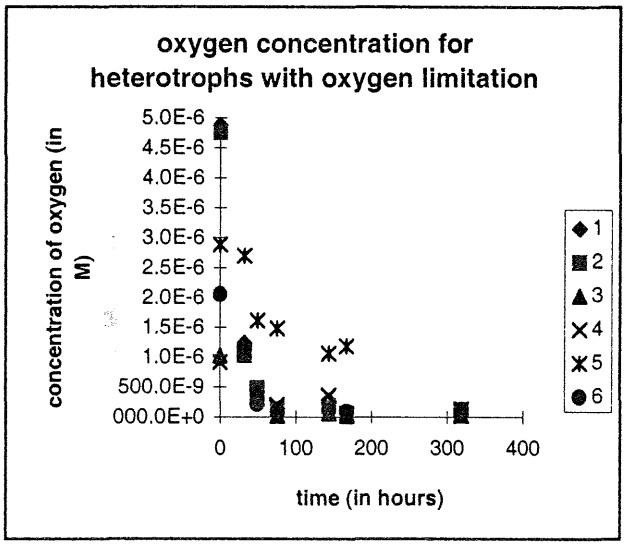


Figure 16: Concentration of oxygen in mol per liter for heterotrophs with oxygen limitation

Secondly the consumption rates of oxygen for the heterotrophs with oxygen limitation have been calculated. The consumption rates are high at the start of the experiment (see figure 17). The consumption rates are highest in batches 1 and 2, that have the highest oxygen concentrations. In these batches the difference between the concentrations at time zero and 32 hours is very high and thus the consumption rates should also be high.
Some negative values have been found, which have to be due to measurement errors.

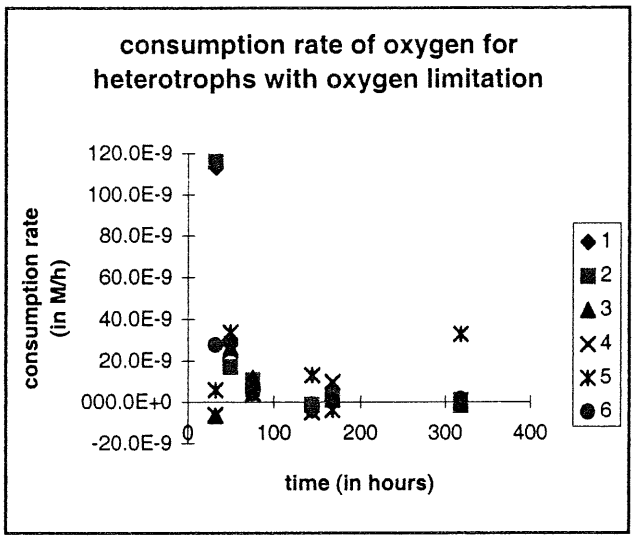


Figure 17: Consumption rate of limited oxygen in mol per liter per hour for the heterotrophs

2.4.6 Acetate concentrations and consumption rates of acetate for the heterotrophs with acetate limitation

This paragraph describes the acetate concentrations and consumption rates for the heterotrophs with acetate limitation. Two strange points have been found in the batches 7 and 8. The concentrations in these batches seem to decrease but after a while they increase again. This seems to be caused by a measurement error.

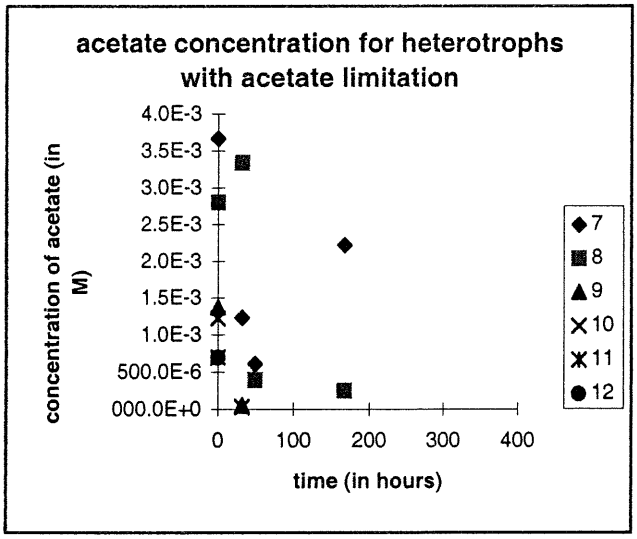


Figure 18: Concentration of acetate in mol per liter for heterotrophs with acetate limitation

Secondly the consumption rates of acetate for the heterotrophs have been calculated.
The batches 7 and 8 show great decline in the consumption rates because of the great difference between the concentrations at time zero and 32 hours. None of the batches show great changes in

the consumption rates after 50 hours.
The negative value of the consumption rate in batch 8 seems to be caused by a measurement error. A more intensive sampling would have been needed for a good estimation of the consumption rate of acetate.

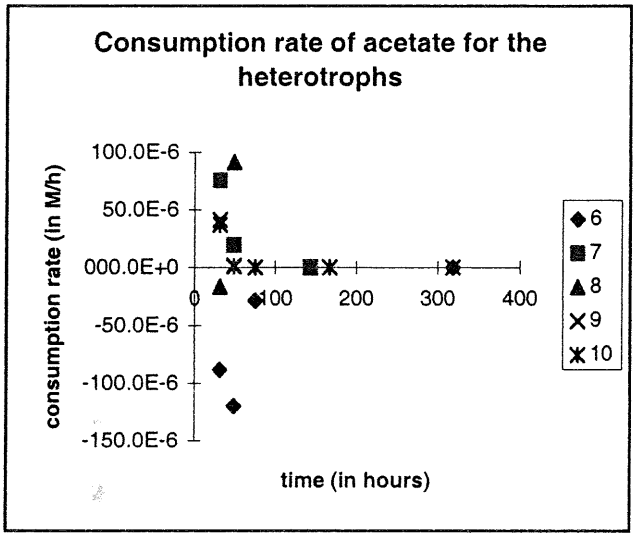


Figure 19: Consumption rate of limited acetate in mol per liter per hour for heterotrophs

Most of the concentrations of the substrates are very low for growth to occur, when the half saturation constants found in the literature are taken into account. However there has been still some growth of the methanotrophs with oxygen or methane limitation and of the heterotrophs with acetate or oxygen limitation. This indicated low values of the half saturation constants for the different substrates.

2.4.7 Calculation of Monod parameters for the experiment

For the determination of the Monod parameters, relative growth rates have to be plotted against the concentrations of the limiting substrate. From these data, the maximum relative growth rates and the half saturation constants have been calculated. The relative growth rate is assumed to be determined by these parameters and the concentrations of the limiting substrate. Therefore these parameters are important for the determination of the growth of the bacteria. Depending on the values for these parameters the outcome of the competition between the methanotrophs and the heterotrophs will vary.
The maximum relative growth rate and the half saturation constant have been calculated by a non-linear optimalisation program which fitted the best curve through the data with the Monod equation.
The data could also be fitted with the Hofstee-plot or the Lineweaver and Burk plot (see appendix B). When the data of the methanotrophs with oxygen limitation have been fitted with the Hofstee equation the points varied much in the y-value (relative growth rate) but there was not much variation in the x-value (relative growth rate divided by the concentration of the substrate). So, small differences have been found between the change in the relative growth rate and the change

in the concentration of oxygen (see also figure 4). Negative values have been found because of decrease of the biomass in time. The data has also been fitted with the Lineweaver and Burk equation. With the Lineweaver plot a scattering of the points has been found. However the r^2 coefficient for determination is very low and the kinetic parameters calculated from this plot are very different than from the ones found with the non-linear optimalisation program.
The data with methane limitation fitted with the Hofstee-equation are clustered at the y-axes (relative growth rates), with equal points above as beneath the calculated line. Again small differences have been found between the change in the relative growth rate and the change in the concentration of methane. A very low r^2 coefficient has been found because only few relative growth rates have been found at higher substrates concentrations.
When the data of the heterotrophs with oxygen limitation has been fitted with the Hofstee plot most of the points are clustered at (0,0) and just few points have a high relative growth rate. This is why a positive relationship has been found. With the Lineweaver plot the data is more scattered. However the r^2 coefficient for determination is very low.
There has been not enough data on acetate limitation to be fitted well with the Hofstee-equation. A high scattering of the data points has been found.
Maybe the initial concentrations have been chosen too low for the bacteria to grow at the maximum relative growth rate.

A comparison between the data and the calculated fit is given for the methanotrophs in figure 20 (oxygen limitation) and figure 21 (methane limitation).
Data of the relative growth rates have been corrected for negative concentrations of the biomass, because those concentrations are caused by calculation errors. These concentrations have been set at zero. Thereafter the relative growth rates have been calculated. Averaged concentrations of the substrate have been used except for the methanotrophs with oxygen limitation, because of a higher r^2. For the determination of the kinetic parameters the maximum relative growth rate has been set at 0.08 (h^{-1}). This has been done because not enough data was available at higher concentrations than $5.0*10^{-7}$ M and therefore a maximum relative growth could not be calculated. The value of 0.08 has been found in literature for the methanotrophs (Gottschal, 1993).
Data from the batches 7 and 8 have not been used, because the Monod equation could not describe the results of these batches. This is the reason that the relative growth rates at the y-axis in figure 20 are different from the relative growth rates in figure 6.
The fitted data for the oxygen limitation for the methanotrophs have a very low r^2 of 0.047 and have a significance at $P > 0.1$. This value is very low because of the clustering of the data. This means that the equation is not significant and thus that the experiment will have to be redone for a better estimation.

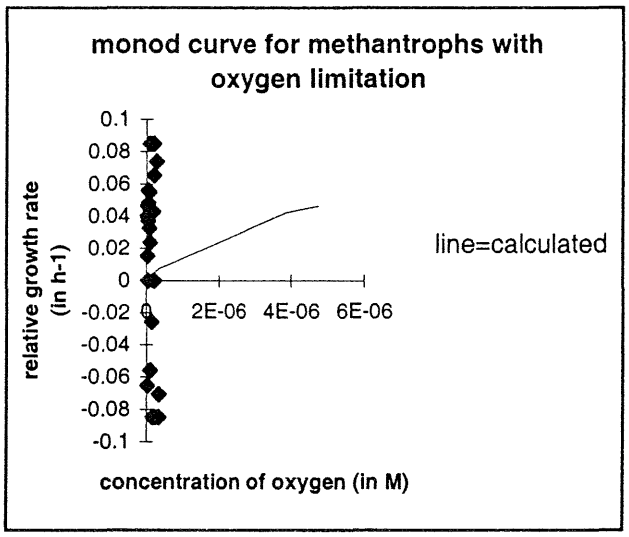


Figure 20: Monod plotted for methanotrophs with oxygen limitation

Data used for the determination of the kinetic parameters with methane limitation are relative growth rates calculated from the biomass concentrations with optical density measurements. At time zero and 24 hours biomass concentrations measured with the Bürker-Türk counting-chamber have been used. This has been done because it was thought the relative growth rates are too high at the start of the experiment for the measurements with the optical density method. While hardly any growth has been found in the first 50 hours. This is the reason that the relative growth rates at the y-axis vary with the relative growth rates in figure 5.

A wide range of relative growth rates have been found at concentrations of less than $4*10^{-4}$ M of methane with methane as the limiting substrate. Just two points have been found at higher substrate concentrations at time zero in the batches 1 and 2. However the calculated data give a low value of the maximum relative growth rate and even lower when more weight has been given to the points with higher substrate concentrations. Therefore the maximum relative growth rate has been set at 0.08, so that this value should not be based just on two points. Besides the value should be in the same range as the maximum relative growth rate found for limiting concentration of oxygen. The maximum relative growth rate depends not on the type of substrate but of the bacterium itself. It should thus not make any difference whether the maximum relative growth rate is determined for limiting methane or for limiting oxygen concentrations.

The fitted line for the methane limitation has a r^2 of 0.17 and a significance at $P > 0.1$. This also means that the equation is not significant and thus that the experiment will have to be redone for a better estimation.

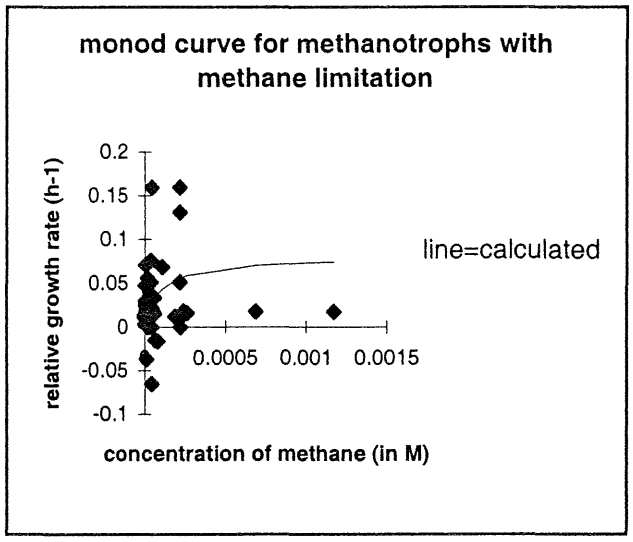


Figure 21: Monod plotted for methanotrophs with methane limitation

For the heterotrophs a comparison between the data and the fits is given in figure 22 (oxygen limitation) and figure 23 (acetate limitation). The data with a oxygen limitation does not contain the data of batch 5, for the reason that the Monod equation could not describe the results of batch 5. The fitted data for oxygen limitation have a r^2 of 0.55 and a significance at $P < 0.02$. So the equation is significant. The values of the kinetic parameters that have been calculated are μmax = 0.32 (h^{-1}) and Ks = $1.2*10^{-5}$ (M). Both values have relative high standard errors of 0.61 and $2.7*10^{-5}$ respectively. The clustering of the points around the y-axes could be responsible for these errors.

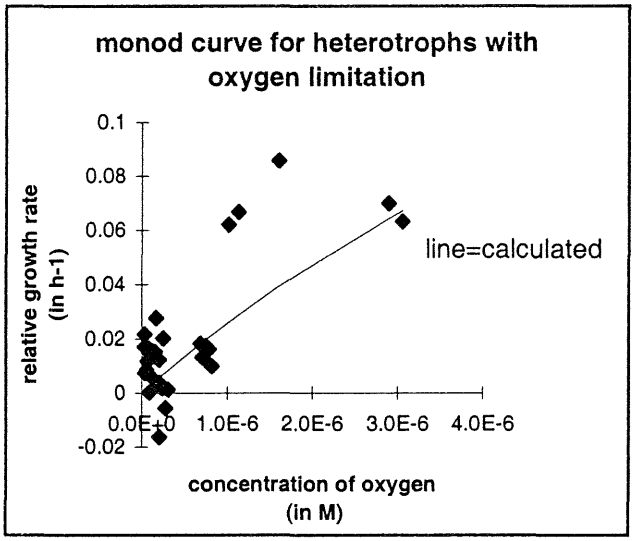


Figure 22: Monod plotted for heterotrophs with oxygen limitation

The data of the heterotrophs with acetate limitation could not be described well by the fitted curve. The data with limiting acetate are few in number and scattered. The coefficient of determination is 0.32 and the fitted data have a significance at $P > 0.1$. This means that the equation is not significant and thus that the experiment will have to be redone for a better estimation. The maximum relative growth rate found (0.05 h^{-1}), is not in the same range compared to the value for oxygen limitation (0.32 h^{-1}). The value of the half saturation constant ($2.56*10^{-3}$ M) is in the same range as found in literature.

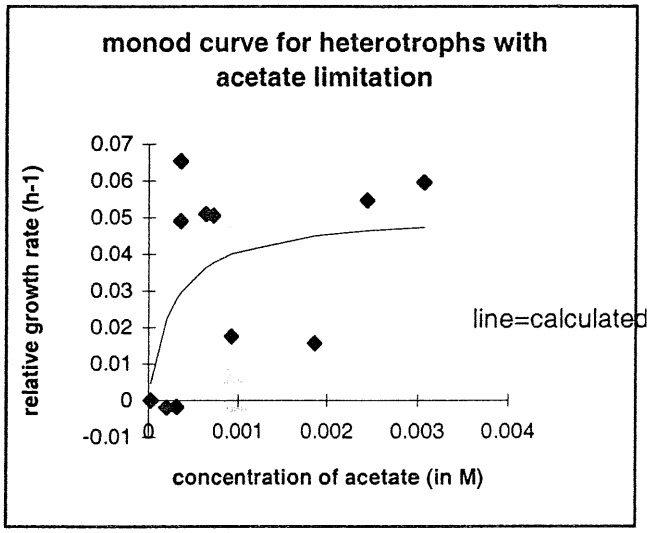


Figure 23: Monod plotted for heterotrophs with acetate limitation.

2.4.8 An overview of the Monod parameters of the experiment.

In this paragraph an overall view is given of the most important Monod parameters of the experiment. The values of the Monod parameters found in literature for the relative growth rates are 0.08 (h^{-1}) for the methanotrophs and the extreme values found for the heterotrophs are 0.036 (h^{-1}) and 0.39 (h^{-1}). In the experiment the value for the methanotrophs with oxygen and methane limitation has been set at 0.08 (h^{-1}). However the equations are not significant for the two types of limitations for the methanotrophs. The value for the heterotrophs with oxygen limitation calculated is 0.32 (h^{-1}) and with acetate limitation calculated is 0.05 (h^{-1}). The differences in the maximum relative growth rates for the different substrate limitations for the heterotrophs are very high. Because the maximum relative growth rates are independent of the types of substrate these differences should be very low. However the equation for the heterotrophs with acetate limitation with acetate limitation is not significant, so not much could be said about this difference in maximum relative growth rate. Only a value for the relative growth rate with oxygen limitation for the heterotrophs has been calculated. This value lies within the range of the values found in the literature.
The values of the half saturation constants for the methanotrophs found in the literature are $3.3*10^{-5}$ M with oxygen limitation and $5.15*10^{-5}$ M with methane limitation. For the heterotrophs the values are $1.38*10^{-6}$ with oxygen limitation and $1.56*10^{-3}$ with acetate limitation. The results of the experiment for the half saturation constants for the methanotrophs are $3.45*10^{-6}$ with

oxygen limitation and $9.5*10^{-5}$ with methane limitation. For the heterotrophs have been calculated a value of $1.53*10^{-6}$ with oxygen limitation and $2.56*10^{-4}$ with acetate limitation. Thus a lower value has been calculated for the methanotrophs with oxygen limitation and higher values for the methanotrophs with methane limitation and the heterotrophs with oxygen and acetate limitation. However it can be questioned whether those values with the errors taken into account are really significant different from those found in the literature.
Still the concentrations of the substrates are lower than the values of the half saturation constants calculated, while still growth has been found. An explanation could be the hypothesis that at lower substrate concentrations the maintenance coefficient decreased and therefore more substrate has been consumed for growth (Panikov, 1995). Another explanation for the absence of growth in the first 50 hours could be a low activity of the bacteria at the start of the experiment due to bad circumstances.
For the methanotrophs with methane limitation low values of the biomass have been found. This is probably caused by the low concentrations of oxygen in these batches.
Very low consumption rates of oxygen have been measured in comparison to the consumption rates of methane and acetate. This seems strange because more oxygen than methane or acetate is needed for growth, so the consumption rates of oxygen have to be higher than of methane and acetate. Maybe an explanation for the low consumption rates of oxygen is the death volume of the needle of the syringe that injects in the headspace of the bottle each time samples have been taken for the measurements. Then the concentrations of oxygen do not change very much and thus the consumption rates are very low.
The calculated values of the Monod parameters have relatively high standard errors, due to the less variation in the data and the few data that are available. Therefore the results have to be compared to other values calculated in other experiments and found in literature for the same conditions.

Table 5: Monod parameters of the experiment for methanotrophs and heterotrophs

	substrate	Ks (mol/liter)	std. Error	mumax (1/h)	Std. error
Methanotrophs	Oxygen	$3.45*10^{-6}$	$1.1*10^{-5}$	0.08	
	methane	$9.5*10^{-5}$	$4.9*10^{-5}$	0.08	
Heterotrophs	Oxygen	$1.2*10^{-5}$	$2.7*10^{-5}$	0.32	0.61
	Acetate	$2.56*10^{-4}$	$3.36*10^{-4}$	0.05	0.018

2.4.9 Conclusion

From the experiments it can be concluded that growth of the biomass of the methanotrophs was higher than of the heterotrophs, due to a higher relative growth rate. Nothing could be said about the maximum relative growth rates and the half saturation constants, because the equations are not significant. Except for the equation of the heterotrophs with oxygen limitation which is significant. The maximum relative growth rate lies in the range of values found in literature which varies from 0.036 to 0.39. However the high standard error has to be taken into account when using this value. The half saturation constants are in the same range as found in literature if the standard errors have been taken into account. The half saturation constants have to be very low for the consumption of oxygen to occur. Such low values of the half saturation constants for oxygen have hardly been described in literature for pure cultures (see § 4.3).

Chapter 3 BOM experiment

3.1 Introduction

With BOM (Biological Oxygen Monitoring Measurements) experiments maximum consumption rates of substrates can be measured for example pure cultures of bacteria.
The experiments can also be done with soil slurry or mixed cultures to measure the competition between bacteria.
The BOM have a short time period of the experiment and therefore the repeatability of the measurements is high. Also a lot of hypotheses can be tested with this method.
In this study, the BOM was used as a control on the kinetic parameters obtained from the batch experiment for pure cultures. The aim was to measure maximum relative growth rates and maximum consumption rates. This was only done for acetate limitation for heterotrophs. Oxygen limitation is only possible if the chamber of measurement is free of leakages of oxygen. It was not sure whether this was the case. Besides there was a problem with the BOM experiment and therefore further experiments with acetate and methane limitations were not performed. The problem was that the concentration of oxygen decreased very slowly. So it would be very difficult to measure the concentration of oxygen for low concentrations of the limiting substrates, because the distinction between the use of oxygen by the bacteria and by the electrode could hardly be made.

3.2 Material and methods

The concentration of oxygen (in %) in the culture of the heterotrophs has been measured with an oxygen electrode. The electrode for oxygen is placed in a closed chamber in which the liquid stirs continuously. The closed chamber is also connected to a water bath, so that the temperature in the closed chamber is kept constant. The measurement equipment is attached to a recorder, therefore the measurement can be monitored continuously and the concentration of oxygen has been measured continuously.
Each day starts with a calibration. This can be done in two ways. The first way is stirring demi water with compressed air. This stirring has been done in a second water bath with the same temperature as the first one. The air saturated water has been measured with the BOM measurements and the deflection of the measurement equipment is set at 100. In that case the result of the recorder has to be 100 also. The adjustment of the measuring equipment has to be fixed by 'lock'.
The second method is almost the same, but then demi water has been stirred with 100% oxygen. The second one is easier for the calculation of the concentrations in (M), but the course is less sensitive to measure. After the calibration, the measurement of the maximum consumption rate has been done. This has been done by measuring the concentrations of oxygen with the electrode in time. The consumption rates of oxygen can be calculated by differentiation of the concentrations in time. The rate of oxygen consumption is proportional to the rate of the consumption of acetate.
The measuring of the decrease in the concentration of oxygen has been repeated nine times for two different acetate concentrations and two different amounts of heterotrophs. Then a curve with the (ln) concentrations of oxygen against the time has been made to calculate the maximum

consumption rate at t=0. The general set up of the BOM measurements is given in figure 24:

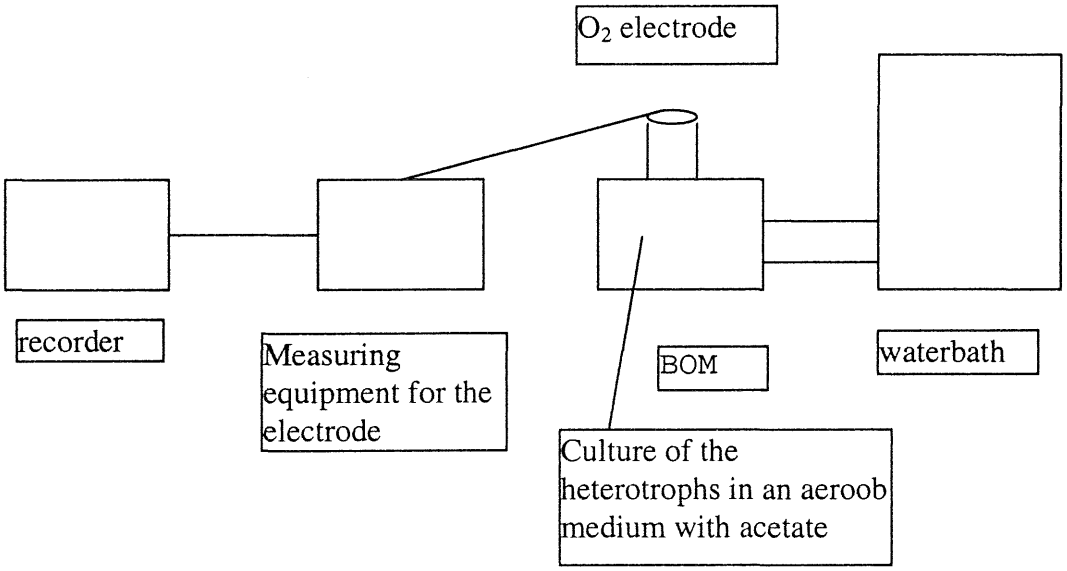


Figure 24: General set up of the BOM experiments

All measurements have been performed at a constant temperature of 20 °C. At 20 °C the solubility of oxygen in the liquid phase is 0.03 ((mol per liter in the gas phase) / (mol per liter in the liquid phase)) (Wilhelm et al. 1977). The concentration of oxygen in air is 8.65 mol/m^3, calculated with the gas law.
Another condition is that the same culture with the same optical density has been constantly used. In case that this has not been the case the optical density has been measured first with a photospectrometer at 660 nm.

Assumptions that has to be made for the calculation of the kinetic parameters is that 1 bacterium weighs $1.1*10^{-13}$ g (Bratbak, 1985) and 1 g bacterium contains 0.57 g carbon (Schlegel, 1972). The yield factor for the pure culture of heterotrophs is assumed to be 0.61 mol bacterial carbon per mol carbon consumed (Spivak, 1994). In the experiment the biomass of the bacteria is assumed to be constant because of the short period for growth.

Measurement and calculation of maximum consumption rate

A sterile medium has been made without substrate. This has been injected into the cell for measurement and then the desired concentration of acetate has been injected. After inserting the electrode into the cell, the measuring lasts until a stable signal has been reached. Then a heterotrophic culture has been added with a syringe. The course of the signal in time has been measured. The time of the measurement is not known beforehand, because it depends on the activity of the culture (between 20 - 30 minutes).
The concentrations of oxygen (in %) have been ln-transformed and have been plotted against time. Then the linear coefficient has been calculated at time zero for each concentration of

acetate. This has been done at time zero, because at that time the consumption rate is maximum and at that moment the values of the other state variables are also known exactly. The maximum consumption rate of oxygen has been calculated from the linear coefficient multiplied by the concentration of oxygen at time zero for each concentration. The maximum consumption rates have been averaged for each concentration of the bacteria.
At the end of the measurement, the cell has been pumped dry with a water pump and the measurement could be repeated with a different substrate concentration.

Calculation of the maximum relative growth rate

The bacterial concentration has been measured at time zero with optical density measurements and has been transformed to a number of bacteria per liter with the linear relationship found in paragraph 2.4. The concentration of bacteria has been corrected for the number of µl of bacteria added at time zero.
The specific activity (Qmax in mol carbon consumed/mol bacterial carbon*h) has been calculated by the maximum consumption rate divided by the biomass concentration. The maximum relative growth has been calculated from the specific activity multiplied by the yield factor.

3.3 Results of the BOM experiment

The maximum consumption rate for 150 µl of a known concentration of bacteria added, is $7.5*10^{-3}$ % O_2 / s. This has been converted to a maximum consumption rate in mol O_2 / (liter * s) with the concentration of oxygen found in the liquid for 100 % of oxygen. The maximum consumption rate for 250 µl of a known concentration of bacteria added, is
0.014 % O_2 / s. The concentration of bacteria that has been measured with the optical density measurements is 0.193.
In the table below an overview is given of the results of the maximum consumption rates and the specific activities and the maximum relative growth rates for both concentrations of bacteria. The maximum consumption rates have not been corrected for the change in volume due to the amount of bacterial solution.

Table 6 : Results of the BOM experiment of maximum consumption rate, specific activities and maximum relative growth rates for different amounts of bacteria added.

ml of bacteria added	Vmax (molC/(liter*h))	Qmax (molC/(molC*h))	mumax(h^{-1})
150	$7.78*10^{-5}$	$3.67*10^{-3}$	$1.08*10^{-2}$
250	$1.47*10^{-4}$	$4.15*10^{-3}$	$1.21*10^{-2}$

If these results are compared with those from the batch experiment, it is found that the maximum consumption rate is in the same range as found in the batch experiments. The specific activity (Qmax) is however higher for the batch experiments. This is maybe due to the higher biomass estimations in the batch experiment. With the same yield factor and a higher specific activity a higher maximum relative growth rate can be expected in the batch experiment. The

batch experiment shows that the maximum relative growth rates for the heterotrophs are 5 times higher than in the BOM experiment. Taking the differences in the amount of biomass into account the maximum relative growth rate calculated from the BOM experiment is probably similar to the maximum relative growth rate with acetate limitation calculated from the batch experiment.

Chapter 4 Model about competition for oxygen between the bacteria

4.1 Introduction

The model presents a simplified representation of the competition for oxygen between methanotrophic and heterotrophic bacteria in a rhizosphere (see Appendix D for the source code). The competition model has been developed to give an impression of the influence of the methanotrophs on the methane oxidation for saturating values of the substrates and for concentrations of the substrates in the rhizosphere. Wherefore a set of parameters has been used from the literature. The same model but without competition with a set of parameters from the batch experiment has also been used to compare the results of this model with the results of the experiments. With the model some insight was obtained in the sensitivity of the results for the variation of the values of the different parameters.
Numerous choices and assumptions have been made during the development of the model.
The choices and assumptions are reflected in the differential equations (see § 4.2).
The state variables distinguished are: Methanotrophs, Heterotrophs, Oxygen, Methane and Acetate. There are three main types of equations: first order rate equations for biomass, double Monod equations for relative growth rates and the Pirt equation for substrate and electron acceptor consumption.
An account of the choices and assumptions that have been made during the development of the model is presented below on the basis of the differential equations.

4.2 Model description

Bacteria

Two groups of aerobic bacteria are considered, i.e. Methanotrophs and Heterotrophs. The growth of the methanotrophs depends on methane and oxygen and the growth of the heterotrophs depends on acetate and oxygen. Growth rate of the methanotrophs is proportional to their respective amount of biomass (van Veen and Frissel 1989; Schlegel, 1972). Thus, it is assumed that the population density of the bacterium never limits its growth, as would be presented by the logistic growth equation (Schmidt et al., 1985). Relative growth rates depending on the concentrations of carbon (methane or acetate) and electron acceptor, have been calculated by double Monod kinetics (Megee et al. 1972; Shah and Coulman 1978; Bader et al. 1975; Bader 1978).

For the methanotrophs: mum = mumaxm*(ox/(ox+ksoxm))*(me/(me+ksme))

and for the methanotrophs: muh = mumaxh*(ox/(ox+ksoxh))*(ac/(ac+ksac))

(see for the equation and the dimensions of the parameters in appendix C and for an explanation of the parameters in §2.2). The double Monod model assumes that the electron-donor (substrate) and the electron-acceptor (oxygen) combine in the same organism. This assumption is based upon the existence of different enzymes that catalyse the respective reductions within the same organism (Knowles, 1982).

There are two parameters which describe the loss of bacterial biomass. The first one is the relative death rate and the second one is the maintenance coefficient. The relative death rate is defined as the death rate divided by the number of bacteria present. The maintenance is defined as the energy consumed by the bacteria for functions different than for the production of new cell material. The concept of the maintenance requirement as negative growth or 'endogenous metabolism', introduced by Herbert (1956), seems artificial and indirect. The concept which expresses the requirement in terms of energy substrate used for maintenance is direct and seems preferable (Pirt, 1965). The latter could be transformed to the first one (personal communication by van Bodegom) with a yield factor which has been assumed to be constant. However it is wrong to use both definitions in one model. Maintenance used as endogenous metabolism is mathematically not distinguishable from the relative death rate. Also in the measurements this distinction is hard to make. This is why the relative death has been assumed to be zero and only the maintenance is taken into account in the equation by Pirt (1965) for the substrates (see Appendix E for a further explanation).
Data for methanotrophs found in literature are from the bacterium *Methylocystis* (Gottschal, 1993) and from pure cultures of methanotrophs (Nagai et al., 1973). Data from *Methylocystis* have been used because first most parameters are known from this genus and second because also in this study a pure culture has been used. Data for heterotrophs found in literature are from the bacterium *Pseudomonas chlororaphis* (Bodelier et al., 1997; Tros, 1996). The dilution of the heterotrophs used in the batch experiment contained most of all *Pseudomonas species.*
There are two types of bacteria:
I) bacteria with a high affinity for the substrate and
II) bacteria with a low affinity for the substrate.
High affinity means that the bacteria are capable of growing at low concentrations of the different substrates that are needed for their growth. Low affinity means that the bacteria can grow at high concentrations of the substrates. Therefore different types of bacteria from the same group which diverge in affinity for the substrates can exist. In most references this distinction is not made, so this study will not do it either. However only methanotrophs with a low affinity have been found in the rice soil. All bacteria are assumed to be active and the bacteria are assumed to be homogeneously distributed in the rhizosphere and in the liquid phase of the batch.

Consumption of oxygen, methane and acetate

The consumption of oxygen, methane and acetate are described by an equation by Pirt (1965, 1975). For example the consumption rate of oxygen:

consox = ((mumaxm /Yoxm) + maoxm)*Bm + ((mumaxh/Yoxh) + maoxh)*Bh.

The consumption rates are proportional to the biomass and to the growth rates of the biomass. The first part of this consumption rate represents the use of substrate for cell synthesis and energy for growth. Whereas the second part represents the maintenance requirements of the organism for e.g. turnover of cell materials, osmotic work to maintain concentration gradients between the cell and the surroundings (Pirt 1975) and also death of the bacteria.
All substrates in the model served both as substrates for growth and as energy source for the bacteria. It is assumed that there are no other important electron acceptors than oxygen.

In this model it is assumed that no products of cell decay or mineralisation will partially enter the surroundings, and that no products will be used again as substrates for growth and maintenance. There is no substrate production. For the calculation of the concentrations of oxygen and methane in mol per liter in liquid phase during the simulation the amount of substrate from the headspace that dissolves in the liquid phase is continually taken into account (see Appendix D for the meaning and the dimensions of the parameters).

Tamgo = Vw*Cw + Vg*Ch and Kwgox=Cw/Ch;
Cw = Ch*Kwgox; Tamgo=Vw*Ch*Kwgox + Vg*Ch;
Tamgo = Ch*(Vw*Kwgox + Vg);
Ch = Tamgo/(Vw*Kwgox + Vg).

The above has been done because of the equilibrium that exists between the gas phase and the liquid phase. In the competition model it is assumed there is an equilibrium between the gas phase of the single rice root and the water phase of the soil for oxygen. The concentration of methane is assumed to be already in the water phase. Because rice grows in flooded soils, the gas phase in the soil has been neglected. The volumes of the gas phase in the root and the liquid phase in the rhizosphere in the competition model are from van den Hout. The root and the rhizosphere are assumed to be cylinders. The volume of the gas phase in the root is the volume of the cylinder multiplied with the porosity of the rice root. The volume of the liquid phase is the area of the rhizosphere corrected for the area of the rice root and multiplied by the height of the root. The volumes of the gas phase and of the liquid phase for the model with parameters of the batch experiment have been taken the same as for the batch experiment. The concentration of acetate in the model with initial data from the literature is a concentration measured in the rice soil. The concentrations of oxygen and methane are based upon solubility products found in the literature (Wilhelm et al., 1977).

Carbon balance

The carbon balance is the net concentration of carbon. The consumption of carbon is equal to the growth of the methanotrophs and the heterotrophs and the respiration rate of both bacteria.
To calculate the respiration rate of both bacteria the following has been done (see Appendix D for an explanation of the meaning and the dimensions of the parameters):

The respiration rate of both bacteria = the consumption of carbon of both bacteria - the growth of both bacteria:

(growm/yoxm*stoch)*bm + mame*fact1*bm) + ((growh/yoxh)*bh + maac*fact2*bh) - (growm*yoxm*stoch)/yoxm*stoch - growh*yoxh/yoxh

The respiration rate then becomes:

((growm*(1- yoxm*stoch))/yoxm*stoch)*bm + mame*fact1*bm + ((growh* (1-yoxh))/yoxh)*bh + maac*fact2*bh

This respiration rate is integrated by time, so that the net carbon balance as a fraction of the net concentration of mol carbon per liter and the initial concentration of carbon becomes:

nc = ((itamg*fact1/(va + vw)) + iac*fact2 + ibm + ibh - (tamgm*fact1/(va + vw)) - ac*fact2 - ibm - ibh - c)/(itamgm*fact1/(va + vw)) + iac + ibm + ibh)

Environmental conditions

The environmental conditions in the model have been taken the same as in the batch experiment. The environmental conditions in the references from which parameters were used in the model could be different from the environmental conditions in this model. This has to be taken into account. However for the model with initial data from the batch experiment this problem did not occur. A comparison of the results for a variation of input values of parameters has been done.
A model run stops if either the appropriate electron acceptor (oxygen) or electron donor (methane or acetate) for growth has been depleted or if the simulation time has been reached.
Empirical relationships between microbial activity and soil pH, temperature and water potential are not incorporated in the model. The temperature was set at 30 °C for the condition that a part of the concentration of oxygen and methane in the gas phase dissolves into the liquid phase. This was the same temperature as of the climate chamber in the experiment.

Computer program

Numerical calculations were done by a program written in FST (FORTRAN Simulation Translator). The syntax of the FST language was based on the syntax of CSMP III (IBM, 1975).
This model contains three sections:
I) an initial section, summarising initial conditions of the state variables and all biological and run time control parameters:
II) a dynamic section, starting with the state variables in terms of amounts contained in integrals. The latter is followed by net growth rates, net consumption rates, growth rates and a carbon mass balance:
III) a terminal section, containing finish statements if one of the substrates becomes lower than zero.
Units of variables have been declared in a separate listing within the program. All results presented have been obtained by the variable time step integration method of Runge-Kutta.
During the simulation run amounts of biomass, concentrations of the substrates, net growth rates, net consumption rates, growth rates of the bacteria and the amount of carbon have been computed.

4.3 Literature review of the kinetic parameters

In the literature many different values were found for the parameters for growth of the bacteria. The aim of this paragraph is to review these values for the different methods used for calculation. This list has been be used for the estimation of the parameters in the competition model. A comparison of the values found in the literature has to be made for a good estimation of the parameters in the model. For an explanation of the parameters see §2.2.
The first step in the comparison between the data found, is to define uniform dimensions. The parameters and its dimensions used are:

mumax (maximum relative growth rate) in h^{-1}
Vmax (maximum consumption rate) in mol substrate per liter per hours
Km (Michaelis Menten constant) in mol substrate per liter
Qmax (specific activity) in mol carbon in substrate per mol bacterial carbon per hour
Y (yield factor) in mol bacterial carbon per mol carbon in substrate
m (maintenance coefficient) in mol carbon in substrate per mol bacterial carbon per hour

The specific activity, the yield factor and the maintenance coefficient for carbon in substrate are directly related to these parameters for oxygen, methane or acetate. For the calculation of the yield factor and maintenance coefficients an assumption has been made that 1 g dry weight of bacteria contains 1 g of carbon.
Only Michaelis Menten constants are found in the literature. However some of these values have been used for the calculation of the relative growth rate of the bacteria. This has been done because the distinction between the Michaelis Menten constant and the half saturation constant is not carefully made in the literature.
The tables present the growth kinetic parameters for both bacteria and for the types of substrate limitations (Table 7-11 for the methanotrophs and Table 12-17 for the heterotrophs).

Table 7: Michaelis Menten constants for oxygen for the methanotrophs

Methanotrophs		
for oxygen **Reference**	**Km**	**Assumptions or Remarks**
Frenzel et al. (1990)	$8*10^{-6}$ - $36*10^{-6}$	fresh water sediment cores
Lidstrom and Somers (1984)	$20*10^{-6}$	fresh water sediment
Joergenson (1985)	$0.3*10^{-6}$	*Methylosinus trichosporium*
Megraw and Knowles (1987)	$37*10^{-6}$	humisol preincubated with 20% CH_4 for 6 days
Hardwood and Pirt (1972)	$0.7*10^{-6}$	Methylococcus capsulatus
Gottschal (1993)	$3.95*10^{-6}$ - $5.45*10^{-6}$	50:50, v/v air/methane mixture *Methylocystis*
Watson et al. (1997)	$32*10^{-6}$	

Table 8: Michaelis Menten constants for methane for the methanotrophs

Methanotrophs for methane		
Reference	**Km**	**Assumptions or Remarks**
King (1990)	$2.2*10^{-6}$ - $3.7*10^{-6}$	fresh water sediment
Bender and Conrad (1994)	$41*10^{-6}$	lake sediment
King (1994)	$3*10^{-6}$ - $6*10^{-6}$	sediment free roots
Lidstron and Somers (1984)	$9.5*10^{-6}$	deep freshwater sediment
Bucholz et al. (1995)	$4.1*10^{-6}$ - $9.6*10^{-6}$	deep freshwater sediment
Hardwood and Pirt (1972)	$62*10^{-6}$	*Mythylococcus capsulatus*
Watson et al. (1997)	$57.9*10^{-6}$	vertical profiles of oxygen uptake potentials measured in peat
King (1992)	$1*10^{-6}$ - $92*10^{-6}$	soil and wetland habitats
Kightley et al.(1995)	$37*10^{-6}$	landfill cover soil
King (1990)	$2*10^{-6}$ - $4*10^{-6}$	Danish wetland sediment
Nedwell and Watson (1995)	$14*10^{-6}$ - $44*10^{-6}$	ombrotrophic peatland
Megraw and Knowles (1987)	$66.2*10^{-6}$	cultivated humisol
Takano and Terui (1975)	$60*10^{-6}$	
Linton and Buckee (1977)	$32*10^{-6}$ - $44*10^{-6}$	
O'Neill and Wilinson (1977)	$45*10^{-6}$ - $48*10^{-6}$	
Nagai et al. (1973)	$1*10^{-6}$ - $4*10^{-6}$	pure cultures
Rudd and Hamilton (1975)	$5*10^{-6}$	in lake water
Lidstrom and Somers (1984)	$10*10^{-6}$	in sediments
Bosse and Frenzel (1997)	$4*10^{-6}$	soil planted with rice
Bosse and Frenzel (1997)	$6*10^{-6}$	isolated roots
Bender and Conrad (1992)		air dried paddy soil
Bender and Conrad (1992)	$14*10^{-9}$ - $58*10^{-9}$	preincubated soil I
Bender and Conrad (1992)	$7.07*10^{-6}$ - $8.03*10^{-6}$	preincubated soil II

Table 9: Maximum consumption rates for oxygen and a specific activity for the methanotrophs

Methanotrophs			
for oxygen Reference	**Vmax**	**Qmax**	**Assumptions or Remarks**
Gottschal (1993)		$4.32*10^{-2}$ - $4.56*10^{-2}$	50:50, v/v air/methane mixture *Methylocystis*
Watson et al. (1997)	$1.81*10^{-6}$		
Watson et al. (1997)	$2.52*10^{-4}$		

Table 10: A maximum relative growth rate and maximum consumption rates for methane for the methanotrophs

Methanotrophs for methane			
Reference	**Mumax**	**Vmax**	**Assumptions or Remarks**
Gottschal (1993)	0.08		50:50, v/v air/methane mixture *Methylocystis*
Watson et al. (1997)		$1.29*10^{-6}$ - $1.4*10^{-6}$	methane oxidation potential
Megraw et al. (1987)		$1.03*10^{-3}$	cultivated humisol
			Vmax, in flooded rice soil profiles
Wang et al. (1997)		$0.48*10^{-3}$ - $1.09*10^{-3}$	0 - 1 cm
Wang et al. (1997)		$0.31*10^{-3}$ - $0.5*10^{-3}$	1 - 3 cm
Wang et al. (1997)		$0.2*10^{-3}$ - $0.34*10^{-3}$	3 - 5 cm
Wang et al. (1997)		$0.05*10^{-3}$ - $0.14*10^{-3}$	5 - 7 cm
Wang et al. (1997)		$0.038*10^{-3}$ - $0.11*10^{-3}$	> 7 cm
Bosse and Frenzel (1997)		$1.2*10^{-6}$	soil planted with rice
Bosse and Frenzel (1997)		$6*10^{-5}$	isolated roots

Table 11: Yield factors and maintenance coefficients for the methanotrophs

Methanotrophs			
Reference	**Yield**	**Maintenance**	**Assumptions or Remarks**
Megraw et al. (1987)	0.1		
Megraw and Knowles (1987)	0.77a		drained peat
Nagai et al. (1973)	0.43		various methanotrophic bacteria
Megraw and Knowles (1987)	0.80a		*M. trichosporium OB3b*
Hardwood and Pirt (1972)			*Methylolcoccus capsulatus*
	0.66		CH_4 limited
	0.25		O_2 limited
Bucholz et al. (1995),	0.38		fresh water sediment
Whalen et al. (1990)	0.69b		landfill soil
Lidstrom and Somers (1984),	0.31		
Roslev and King (1995)		$3.21*10^{-3}$	

a = calculated as CH_4 consumption - CO_2 production
b = C in biomass + organic compounds

Table 12: Michaelis Menten constants for oxygen for the heterotrophs

Heterotrophs		
for oxygen **Reference**	**Km**	**Assumptions or Remarks**
		continous culture with *P. Chlororaphis*
Bodelier et al. (1997)	$5.52*10^{-6}$ - $5.74*10^{-6}$	with glucose and airsat. 80%
Bodelier et al. (1997)	$1.36*10^{-6}$ - $1.40*10^{-6}$	airsat. 0%
Bodelier et al. (1997)	$3.93*10^{-6}$ - $5.67*10^{-6}$	with glutamate and airsat. 80%
Bodelier et al. (1997)	$4.75*10^{-6}$ - $4.97*10^{-6}$	airsat. 0%
		Math. model of chemostat with *C. testosteroni*
Gerritse (1992)	$0.35*10^{-6}$	high affinity
Gerritse (1992)	$400*10^{-6}$	low affinity
Gottschal (1993)	$0.45*10^{-6}$	resting cell suspensions of *C. testosteroni*

Table 13: Michaelis Menten constants for acetate for the heterotrophs

Heterotrophs		
for acetate **Reference**	**Km**	**Assumptions or Remarks**
Spivak et al.(1994)		mathematical model of two types of bacteria
	$1*10^{-3}$	*F. aurantia1*
	$3.33*10^{-4}$	*F. aurantia2*
Gerritse et al. (1989)		modelling of mixed chemostat cultures of aer.and anaer. bact.
	$4.3*10^{-6}$	*C. testosteroni*
Tros (1996)	$1.03*10^{-3}$ - $2.09*10^{-3}$	batch experiment with *Pseudomonas*

Table 14: Maximum relative growth rates and maximum consumption rates for oxygen for the heterotrophs

Heterotrophs			
for oxygen **Reference**	**Mumax**	**Vmax**	**Assumptions or Remarks**
Gottschal (1993)	0.39		50:50 air/methane
Gerritse (1992)		$2.45*10^{-3}$	$[O_2]<0.1*10$-6M
			continous culture with *P. chlororaphis*
Bodelier et al. (1997)	0.0116		gluc. airsat. 80%
Bodelier et al. (1997)	0.065		airsat. 0%
Bodelier et al. (1997)	0.0784		glut. airsat. 80%
Bodelier et al. (1997)	0.085		airsat. 0%
Watson et al. (1997)		$4.8*10^{-8}$	oxygen uptake potentials measured in peat
Gerritse et al. (1989)			model chemostat with *C. testosteroni*
Gerritse et al. (1989)	0.036		
Gerritse et al. (1989)		$1.43*10^{-3}$	redox 34mV
Gerritse et al. (1989)		$2.15*10^{-3}$	redox 19mV
Gerritse et al. (1989)		$1.24*10^{-3}$	redox 14mV
Gerritse et al. (1989)		$2.57*10^{-3}$	redox 12mV

Table 15: Maximum consumption rates for acetate for the heterotrophs

Heterotrophs		
for acetate **Reference**	**Vmax**	**Assumptions or Remarks**
		model chemostat with *C. testosteroni*
Gerritse et al. (1989)	0.28	redox 19mV
Gerritse et al. (1989)	$0.13*10^{-3}$	redox 14 mV

Table 16: Specific activities and yield factors for the heterotrophs

Heterotorphs			
Reference	**Qmax**	**Yield**	**Assumptions and Remarks**
Gottschal (1993)	0.269		50:50 air/methane
Gerritse et al. (1989)	0.269		model chemostat with *C. testosteroni*
Gerritse et al. (1989)	0.352		
Gerritse et al. (1989)	0.1		model chemostat
Gerritse et al. (1989)		0.37	with *C. testosteroni*
Spivak et al. (1994)		0.93	average or *F. aurantia1* and *F. aurantia2*
Tros (1996)		0.48	
Pirt et al. (1965)		0.63	*for A. cloacae*
Hadjipetrou et al. (1963)		0.58	batch cultures with O2 as electron accep. for *K. aerogenes*
Hadjipetrou et al. (1963)		0.46	for *P. mirabilis*
Hadjipetrou et al. (1963)		0.28	for *C. freundii*

Table 17: Maintenance coefficients for the heterotrophs

Heterotrophs	
Reference	**Maintenance**
Tros (1996)	$4.29*10^{-2}$
Pirt et al. (1965)	$1.1*10^{-2}$

A large variability of parameters can be concluded from the values given in the tables. In §4.6 a model analysis has been done for the variation of the different parameters.

4.4 Values of the parameters from literature used in the competition model

The initial amount of bacteria has been calculated from this study.
The concentration of oxygen and methane are not-limiting and are based upon the solubility products for these substrates of both bacteria (Wilhelm et al., 1977). The concentration of acetate has been measured in the rice soil.
The maximum relative growth rate of the methanotrophs is of the bacterium *Methylocystis* (Gottschal, 1993) and the maximum relative growth rate of the heterotrophs is an average of the lowest and the highest value found in the literature for heterotrophs (Gerritse et al., 1989;

Gottschal, 1993) (see §4.3).
The half saturation constants for oxygen and methane for the methanotrophs have been taken from literature for pure cultures of methanotrophs (Gottschal, 1993; Nagai et al., 1973). The half saturation constants for oxygen and acetate for the heterotrophs have been taken from literature for *Pseudomonas chlororaphis* (Bodelier et al., 1997 and Tros, 1996).
The yield factor for oxygen for methanotrophs has been calculated from the average of yield factors for carbon by dividing this value by two (Gerritse, 1992; Megraw and Knowles, 1987; Nagai et al., 1973; Hardwood and Pirt, 1972: Bucholz et al., 1995; Whalen et al., 1990 and Lidstrom and Somers, 1984). An average has been chosen because of the high range in these values. The yield factor for carbon has been divided by two because, two times more oxygen than carbon is needed for growth of the methanotrophs. The yield factor for methane is the same as for carbon. The yield factor of oxygen for heterotrophs has been calculated from an average of yield factors for carbon by dividing this value by two (Gerritse et al., 1989; Spivak et al., 1994; Tros, 1996; Pirt et al., 1965; Hadjipetrou et al., 1963). The yield factor for acetate is two times higher than for carbon based upon its formula of the molecule.
The maintenance coefficient for oxygen for the methanotrophs is from Roslev and King (1994) and is equal to the starvation rate divided by the yield factor for oxygen. The maintenance for methane is two times lower than for oxygen, because of the stochiometry. A maintenance coefficient for acetate has been found in literature for the heterotrophs (Tros, 1996 and Pirt, 1973). The maintenance coefficient for oxygen is also two times higher than for acetate, because of the stochiometry.
The parameters from literature used as input for the competition model are the following:

Table 19: Values of parameters used for the model with a set of parameters from the literature

Parameter	Dimension	Methanotrophs	Reference	Heterotrophs	Reference
IB	mol bact.carbon/l	3.97810^{-3}	this study	$4.14*10^{-3}$	this study
IOX	mol oxygen/l	$0.684*10^{-3}$	Wilhelm et al., 1977	$0.684*10^{-3}$	Wilhelm et al., 1977
IME	mol methane/l	$0.165*10^{-3}$	Wilhelm et al., 1977		
IAC	mol acetate/l			$1.5*10^{-3}$	
MUMAX	h-1	0.08	Gottschal, 1993	0.21	Bodelier et al., 1997
KSOX	mol oxygen/l	$4.7*10^{-6}$	Watson et al., 1997	$1.38*10^{-6}$	Bodelier et al., 1997
KSME	mol methane/l	$2.5*10^{-6}$	Watson et al., 1997		
KSAC	mol acetate/l			$1.56*10^{-3}$	Tros, 1996
YOX	mol bact. carbon/ mol oxygen	0.24		0.53	Spivak, 1994
MAOX	mol oxygen/ mol bact. carbon	$1.74*10^{-3}$	Roslev and King (1995)	$2.70*10^{-2}$	
MAME	mol methane/ mol bact. carbon	$8.7*10^{-4}$			
MAAC	mol acetate/ mol bact. carbon			$1.35*10^{-2}$	Tros, 1996

4.5 Model validation

A validation of the model has been done to compare the model results for the different sets of parameters with the results found in literature and in the batch experiment.
First a comparison with literature has been made and second a comparison with the results of the batch experiment has been made.

1) competition model with a set of parameters from literature
During the simulation the methanotrophs grow slower than the heterotrophs, due to the lower relative growth rate. The lower relative growth rate was due to a lower maximum relative growth rate and limiting concentrations of methane. Methane was very soon limiting due to low concentrations of methane. The carbon balance should be zero, but in the model more carbon was consumed than there was present. Maybe the yield factor has been chosen too high, therefore too much substrate was incorporated into the cells (see figure 25).
A comparison can be made of the results of the competition model with literature. Biomass values for pure cultures of heterotrophs are $17.1*10^{-3}$ - $34.2*10^{-3}$ mol bacterial carbon per liter (Bodelier et al., 1997) and $0.483*10^{-3}$ - $4.17*10^{-3}$ mol bacterial carbon per liter (Gerritse et al., 1992). These values are however under different conditions than in the batch experiment. In the model the concentration of the heterotrophs varies around $4.14*10^{-3}$ mol bacterial carbon per liter. For the methanotrophs concentrations have been found of $7.39*10^{-5}$ mol bacterial carbon per liter in the nearest environment of the roots (Bosse et al., 1997) and $0.129*10^{-3}$ mol bacterial carbon per liter in the paddy soil (Bender et al., 1992). In the model the concentration of the methanotrophs varies around $3.97*10^{-3}$ mol bacterial carbon per liter. The biomass does not vary much because of the short growth period in the model.
The relative growth rate of the methanotrophs in the model varies around 7.83*10-2 h^{-1} and of the heterotrophs around 0.103 h^{-1}. Comparing these relative growth rates with the maximum relative growth rates it appears that the methanotrophs have been more limiting in their growth than the heterotrophs.
Maximum consumption rates found in the literature are compared with the consumption rates at time zero found for the model. The value of the consumption rate of methane at time zero in the model ($6.51*10^{-4}$ M/h) is higher than the value found in the literature by Bosse (1997) (see §4.3). However the biomass that has to be taken into account is also higher in the model. The value of the consumption rate of oxygen at time zero in the model ($2.22*10^{-3}$ M/h) is almost the same as the value found for the heterotrophs (Gerritse, 1992). The biomass in the model is also in the same range as found by Gerritse. The consumption rate of oxygen for the methanotrophs can not be compared with values found in the literature because the biomass was not known for these references. The consumption of acetate can not be compared also because of the unknown biomass.

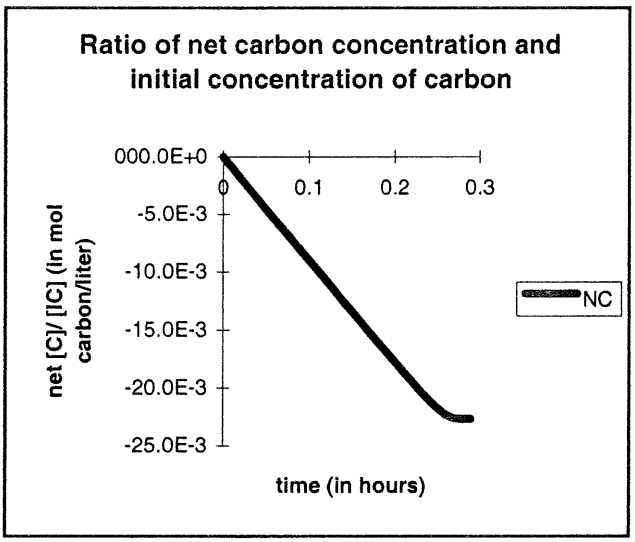


Figure 25: The net concentration of carbon divided by the initial carbon concentration plotted against time

2) model with a set of parameters from the batch experiment.
From the batch experiment the concentrations of the substrates from batch 1 and the calculated half saturation constants have been used for the methanotrophs and the heterotrophs. The other input parameters are the same as for the competition model. The half saturation constants have been used from the batch experiments because these values are in the range as has been found in literature. The maximum relative growth rate for the methanotrophs found in literature was already used in the batch experiment. The maximum relative growth rate for the heterotrophs varied too much for the different types of substrate limitation. Therefore an averaged value of the lowest and the highest maximum relative growth rate found in literature has been used. Yield factors and maintenance coefficients have not been calculated in the experiments, so these values are also from literature.
First the results of the model for the methanotrophs are performed and thereafter the results for the heterotrophs.
The biomass of the methanotrophs hardly changes in time. However in the experiment the biomass did change in time but only after 50 hours. In the model the growth period is very short (2.5 hours), because the concentration of oxygen was limiting.
The relative growth rates of the methanotrophs are in the same range as found in the experiment for the different types of substrate limitations, but decreased faster in the model, due to higher consumption rates of the substrates.
The oxygen in the model is lost in a short time period due to the high consumption rate ($7.86*10^{-4}$ M/h to $6.91*10^{-6}$ M/h) in comparison to the consumption rates found in the batch experiment ($0.2*10^{-6}$ M/h). The consumption rate of methane is also much higher in the model ($3.93*10^{-4}$ M/h) than in the batch experiment ($2.0*10^{-5}$ M/h).
Again more carbon was consumed than was present (figure 26).

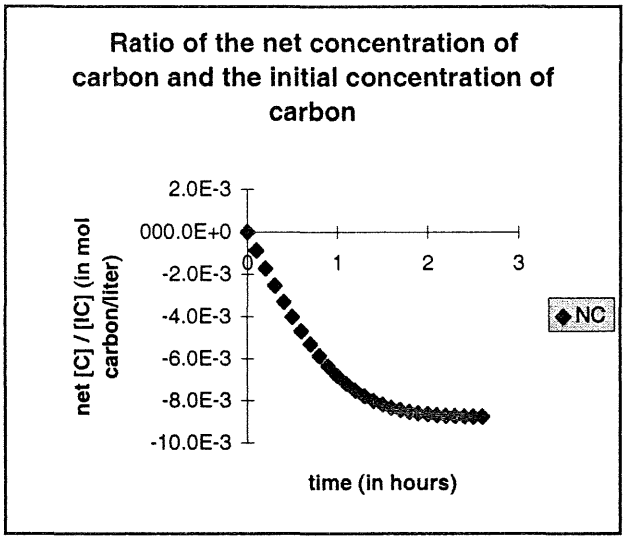


Figure 26: The net concentration of carbon divided by the initial concentration of carbon is plotted for the methanotrophs

The biomass of the heterotrophs does not change much either during the simulation. In the experiment the biomass did change but again only after 50 hours. The period for growth in the model is still very short (1.9 hours) due to oxygen limitation. The relative growth rate is almost the same as in the experiment and is higher than for the methanotrophs. The higher relative growth rate was due to the higher maximum relative growth rate. The consumption rates are much higher for oxygen in the model ($5.65*10^{-4}$ M/h) than in the batch experiment ($0.12*10^{-6}$ M/h). The consumption of acetate in the model is also higher ($2.82*10^{-4}$ M/h) than in the batch experiment ($9*10^{-5}$ M/h). The carbon balance for the heterotrophs is very close to zero (figure 27).
From the comparison of the results of the model with the results of the experiment it can be concluded that the consumption rates of especially oxygen are much higher in the model, while the biomass and the relative growth rates are almost the same in the model and in the experiment. In the next paragraph the different parameters for growth are varied to know the impact of the parameters on the results of the model. Maybe those high consumption rates could be explained in the next paragraphs.

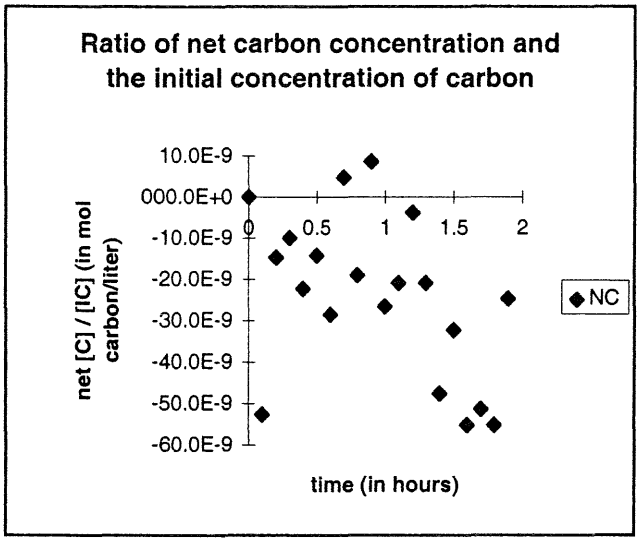


Figure 27: The net concentration of carbon divided by the initial concentration of carbon is plotted for the heterotrophs

4.6 Analysis of the model

The aim of the model analysis was to give some insight in the sensitivity of the results for the variation in the different values of the parameters. An analysis has been done for all the three sets of parameters.
The paragraph describes which parameters have been changed and of their influence on the model results. Also in the figures an overview is given of the results of the changed parameter and of the standard situation. If the results of the changed parameter are similar to the results of the standard situation this has not been showed in the figure.
In the figures 27 – 24 only the concentrations of oxygen and methane have been changed for the competition model with both bacteria taken into account.

$[O_2]$ and $[CH_4]$ (Bedford et al., 1991; Wang et al., 1997; Rothfuss et al., 1993)

Concentrations of the substrates found in the rhizosphere have been used. The concentrations of both oxygen and methane are higher than the standard concentrations. However still methane is limiting and the relative growth rate of the methanotrophs is still lower than the relative growth rate of the heterotrophs. The relative growth rate of the methanotrophs and the heterotrophs increases a few due to the higher concentrations of oxygen and methane for the methanotrophs. The lower relative growth rate of the methanotrophs referring to the heterotrophs is due to the lower maximum relative growth rate and the methane limitation.
The consumption rates of oxygen, methane and acetate increased due to higher relative growth rates. However this increase of the consumption rate of acetate is hardly of influence on the concentration of acetate, which is not limiting.

[O_2] and [CH_4] (Bedford, 1991; Wang, 1997; Rothfuss, 1993)

Here a second value for the concentration of methane found in the rhizosphere has been used which was much lower than the standard concentration. The same concentration of oxygen has been used as above.
Because of the lower value of methane the relative growth rate of the methanotrophs decreased and therefore the consumption rates of oxygen and methane decreased also.

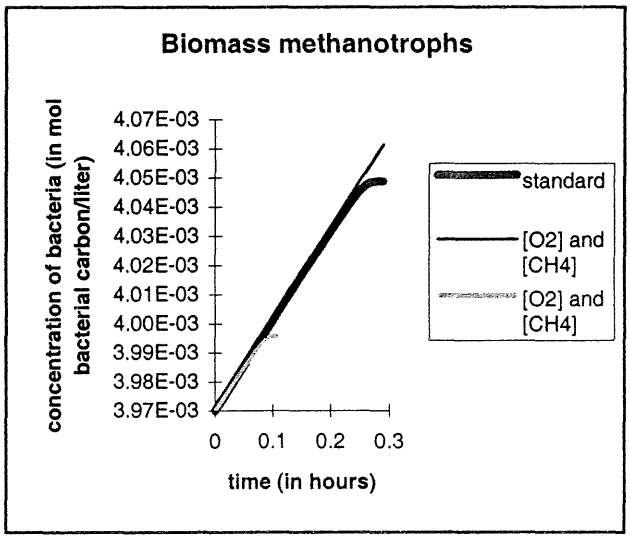


Figure 28: The amount of biomass was plotted for the methanotrophs

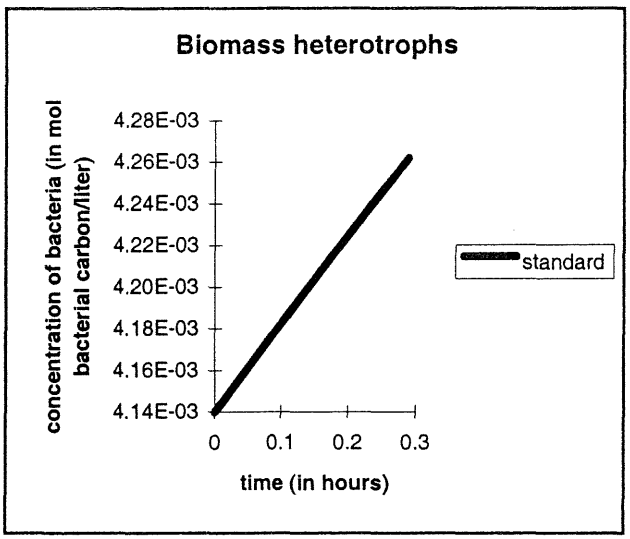


Figure 29: The amount of biomass was plotted for the heterotrophs

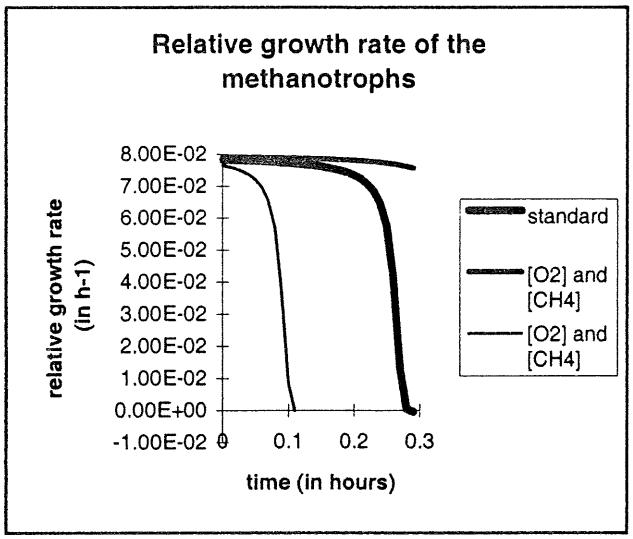


Figure 30: The relative growth rate was plotted for the methanotrophs

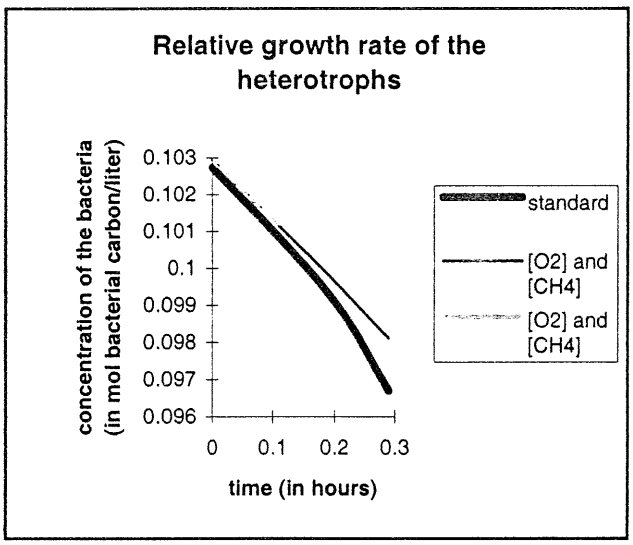


Figure 31: The relative growth rate was plotted for the heterotrophs

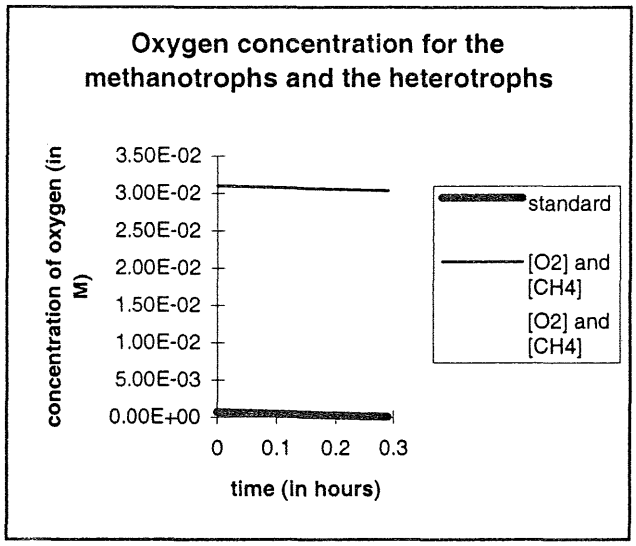


Figure 32: The concentration of oxygen was plotted for the methanotrophs and the heterotrophs

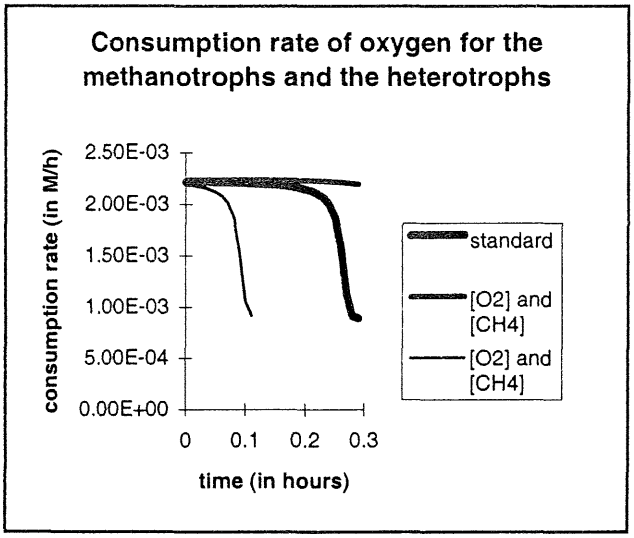


Figure 33: The consumption rate of oxygen was plotted for the methanotrophs and the heterotrophs

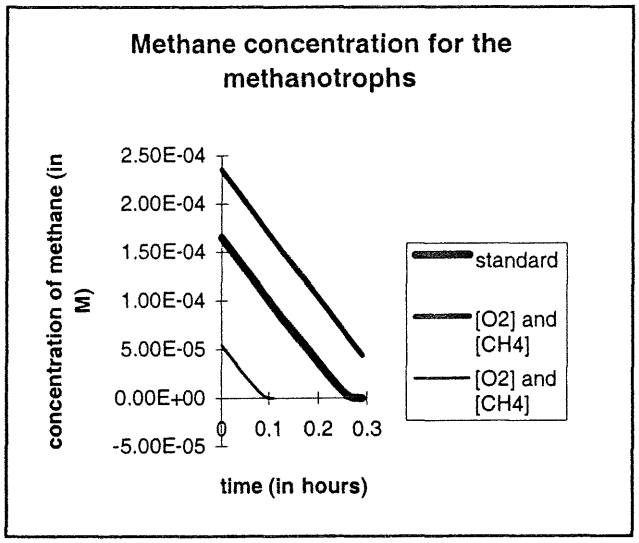


Figure 34: The concentration of methane was plotted for the methanotrophs

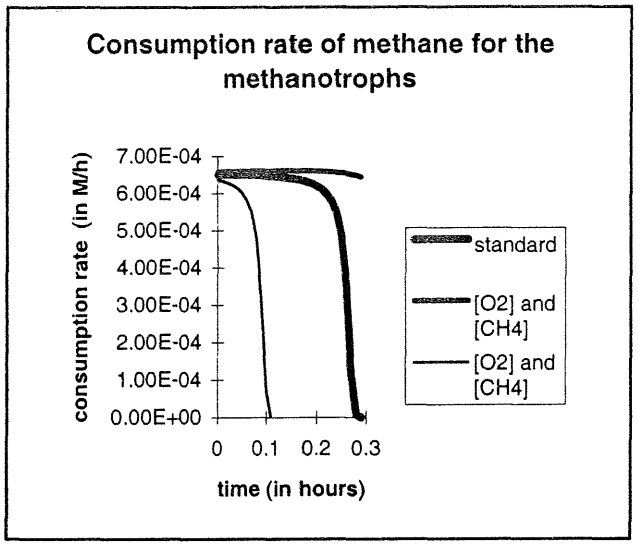


Figure 35: The consumption rate of methane was plotted for the methanotrophs

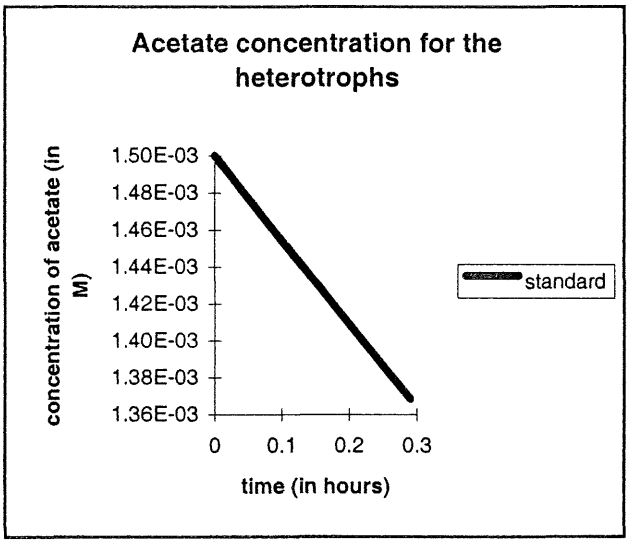


Figure 36: The concentration of acetate was plotted for the heterotrophs

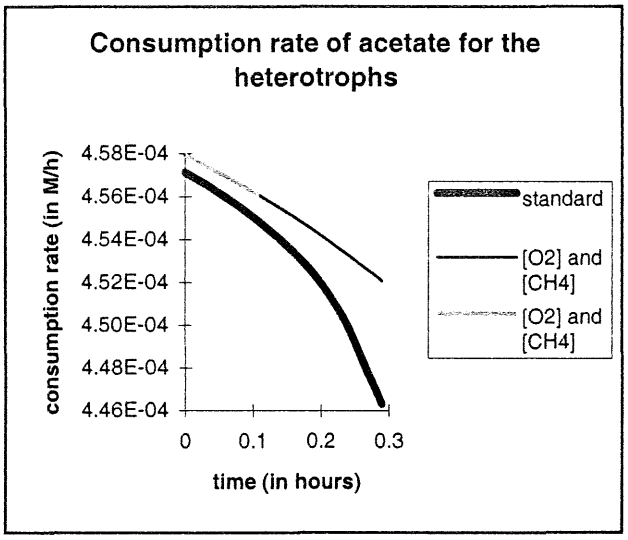


Figure 37: The consumption rate of acetate was plotted for the heterotrophs

Conclusions for the competition model:
When values of the concentrations of oxygen and methane have been used from the rhizosphere methane is still the limiting substrate. Due to the low concentrations of methane and the lower maximum relative growth rate the relative growth rate of the methanotrophs is lower than of the heterotrophs. For these low concentrations of methane and the lower maximum relative growth rate the methanotrophs cannot win the competition for oxygen.

Now a model analysis shall be given of the model with parameters from the batch experiment for the methanotrophs, so no competition has been taken into account.
All kinetic parameters have been changed by a factor two, so the differences in the results could

be compared. Changing the parameters mame = 0., mame = $1.74*10^{-3}$, maoxm = 0., maoxm = $3.48*10^{-3}$,Ksme = $4.75*10^{-6}$ and Yme = 0.24 has no influence on the biomass, the relative growth rate, the concentrations of oxygen and methane and on the consumption rates of oxygen and methane. Except Yme = 0.24 has influence on the concentrations and the consumption rates of methane.

Mumaxm = 0.16

The biomass and the relative growth rate of the methanotrophs increase most by changing the mumax. The concentrations of oxygen and acetate decrease due to the higher consumption rates. The consumption rates of oxygen and methane increase due to the higher relative growth rates.

Ksoxh =$1.73*10^{-6}$

The biomass and the relative growth rate increase due to the higher difference between the concentration of oxygen and the half saturation constant for oxygen. However changing the mumax has more influence on the biomass and the relative growth rate than changing the Ksoxh. The concentrations of oxygen and methane both decrease. After some time when the concentration of oxygen has been decreased to $1.5*10^{-6}$ M, the Ksoxh has more influence on the increase of the consumption rate of oxygen than does the mumax. An explanation for this is that at lower concentrations of oxygen the value of Ksoxh has more influence on the relative growth rate than the mumax.

YOXm = 0.12

The biomass and the relative growth rate decrease due to higher consumption rates of oxygen and methane. The consumption rate of oxygen increases with the same value as for mumax, YOXm* and IBm.

YOXm = 0.12*

In this case only the yield for oxygen has been decreased two times and the yield for methane was kept the same value. The biomass and the relative growth rate decrease with the same value as for YOXm. The concentration of oxygen decrease with the same value as for mumax, YOXm and Ibm, due to the increase of the consumption rate of oxygen. However the concentration of methane is increased by changing this parameter, due to lower concentrations of oxygen and thus lower relative growth rates.

YME = 0.24

Changing this parameter decreases the concentration of methane with the same value as for mumax and IBm, due to an increase of the consumption rate of methane.

$Ibm = 7.94*10^{-3}$

Changing the concentration of the biomass decrease the relative growth rate with the same value as for YOXm and YOXm*, due to the higher consumption rates of the substrates. This results in lower concentrations of the substrates and therefore lower relative growth rates. It has been tried to decrease the biomass to get the same consumption rates for oxygen as has been measured in the batch experiment. However changing the biomass could not solve the problem of the low consumption rates, because at very low values of the biomass, the bacteria stopped growing.

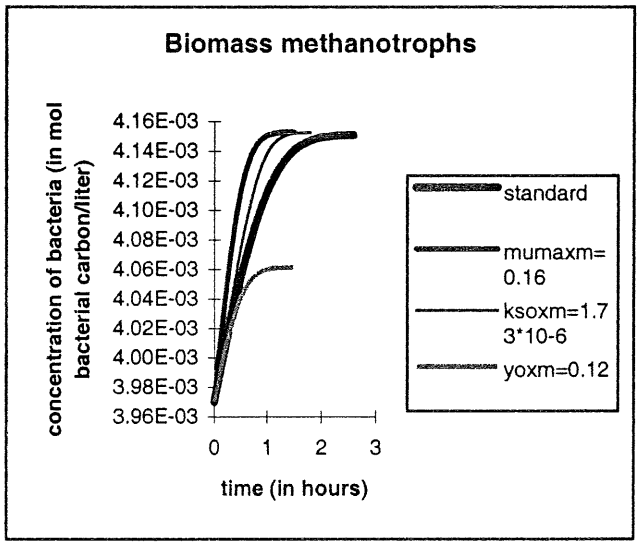


Figure 38: The amount of biomass was plotted for the methanotrophs

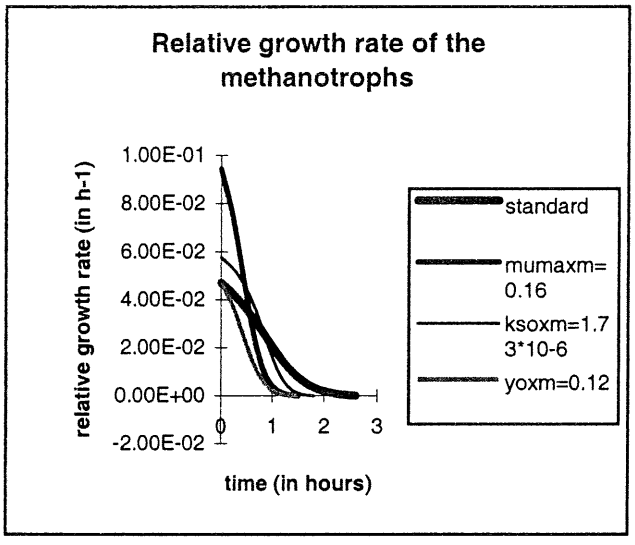


Figure 39: The relative growth rate was plotted for the methanotrophs

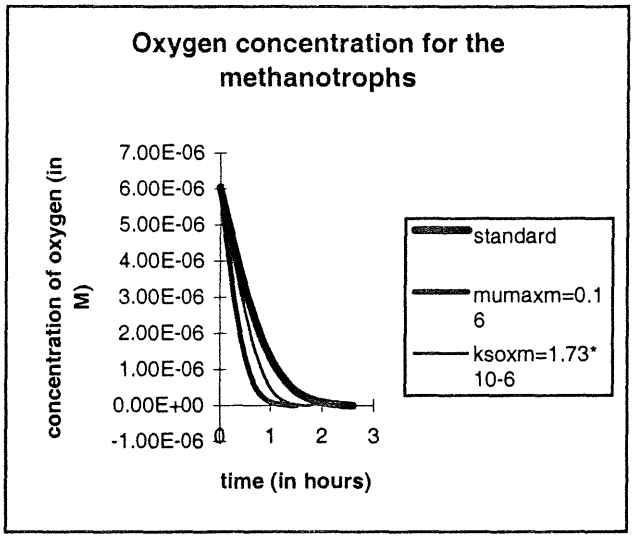


Figure 40: The concentration of oxygen was plotted for the methanotrophs

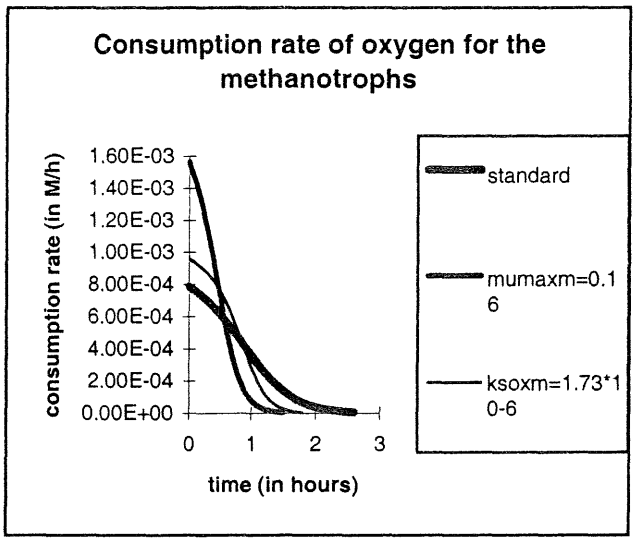


Figure 41: The consumption rate of oxygen was plotted for the methanotrophs

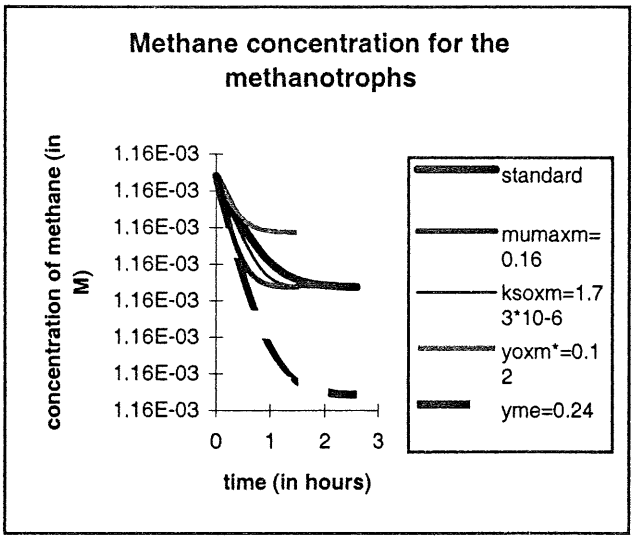


Figure 42: The concentration of methane was plotted a for the methanotrophs

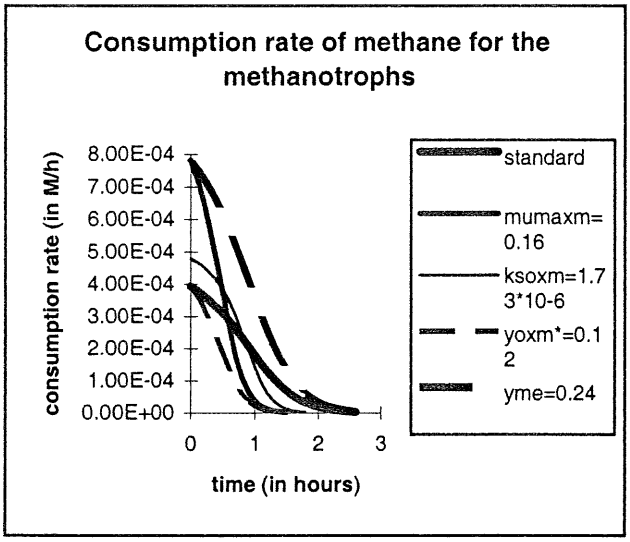


Figure 43: The consumption rate of methane was plotted for the methanotrophs

Conclusions of the model with a set of parameters from the batch experiment for the methanotrophs:
Changing the maximum relative growth rate has most influence on the biomass of the methanotrophs and on the relative growth rates. Changing mumax, Ibm, YOXm and YOXm* increase the consumption rate of oxygen most at higher concentrations of oxygen and at lower concentrations KsOXm has more influence on the consumption rate. Changing the yield factor for methane, the maximum relative growth rate or the biomass have most influence on the consumption rate of methane.
The maintenance coefficients for oxygen and methane and the half saturation constant for methane does hardly change the results of the model.

An analysis shall now be given of the model with parameters from the batch experiment for the heterotrophs, so again no competition is taken into account.
All kinetic parameters were changed by a factor two, so that the differences in the results could be compared. Changing the parameter, KsAc $=1.28*10^{-4}$ has no influence on the biomass, the relative growth rate, the concentrations of oxygen and acetate and on the consumption rates of oxygen and acetate. Changing the parameters maac = 0., maac = $2.7*10^{-2}$ and Yac = 0.53 has only influence on the concentration of acetate and the consumption rate of acetate.

Mumaxh = 0.42

The biomass and the relative growth rates increase most by changing mumax. Changing mumax decreases the concentrations of oxygen and methane due to the higher consumption rates of these substrates.

KsOXh = $6.0*10^{-6}$

The biomass and the relative growth rates increase due to higher consumption rates. At lower concentrations KsOXh has more influence on the increase of the consumption rates of oxygen and acetate.

YOXh = 0.27

Decreasing the yield factor decreases the biomass and the relative growth rate. The consumption rate for oxygen and acetate increase with the same value as for mumax and YOXh*.

YOXh = 0.27*

In this case only the yield factor for oxygen has been decreased by a factor two. The yield factor for acetate has been kept the same value. The biomass and the relative growth rate decrease with the same value as for the parameter above. The only difference is the increase of the concentration of acetate due to the higher consumption rates of oxygen.

Yac = 0.53

This parameter decreases the concentration of acetate with the same value as for mumax, due to higher consumption rates.

MaOXh = $5.4*10^{-2}$

The biomass and the relative growth rates decrease. The concentrations of oxygen decrease due to higher consumption rates. The concentration of acetate increases due to the lower relative growth rates.

MaOXh = 0.

The consumption rate of oxygen decreases, therefore the concentration of oxygen increases and thus the relative growth rate increases. Due to the greater difference between the concentration of oxygen and the half saturation constant for oxygen the relative growth increases and thus also the consumption rate of acetate. When Maac is set at zero, this has no influence on the relative growth rate because the difference in value between the concentration of acetate and the half saturation constant for acetate was high enough. So referring it can concluded that oxygen is the limiting substrate.

*MaAc = 2.7*10-2*

Changing this parameter decrease only the concentration of acetate due to an increase of the consumption rates.

MaAc = 0.

Only the concentration of acetate increase due to lower consumption rates of acetate.

$Ibh = 8.28*10^{-3}$

Changing the biomass increases the consumption of oxygen and acetate most of all. The relative growth rate decreases due to higher consumption rates, thus lower concentrations and therefore lower relative growth rates.

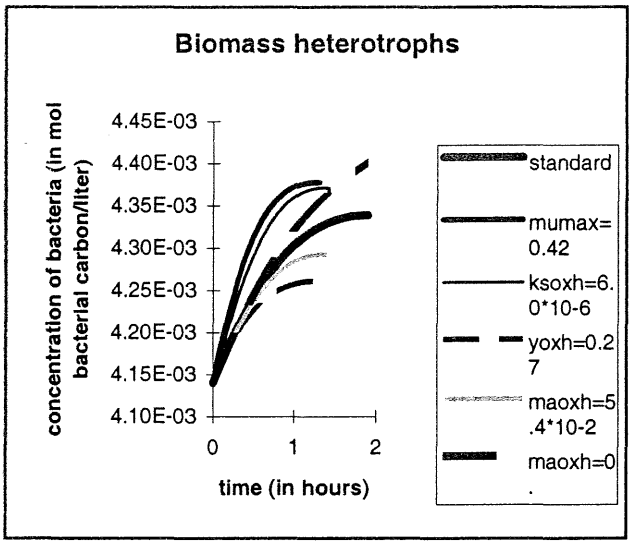


Figure 44: The amount of biomass was plotted for the heterotrophs

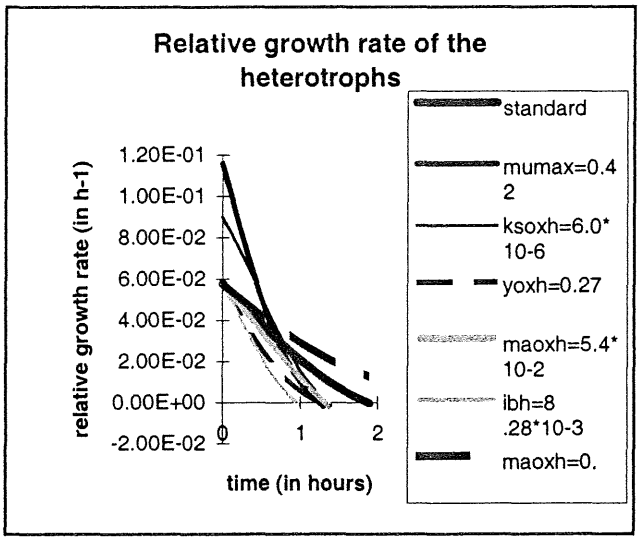


Figure 45: The relative growth rate was plotted for the heterotrophs

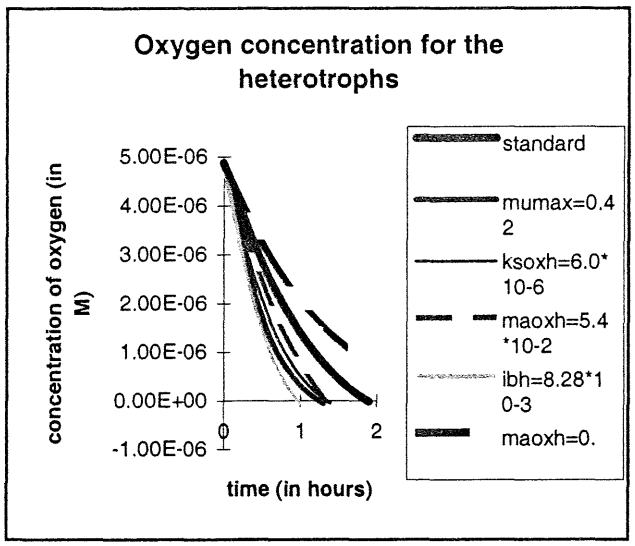


Figure 46: The concentration of oxygen was plotted for the heterotrophs

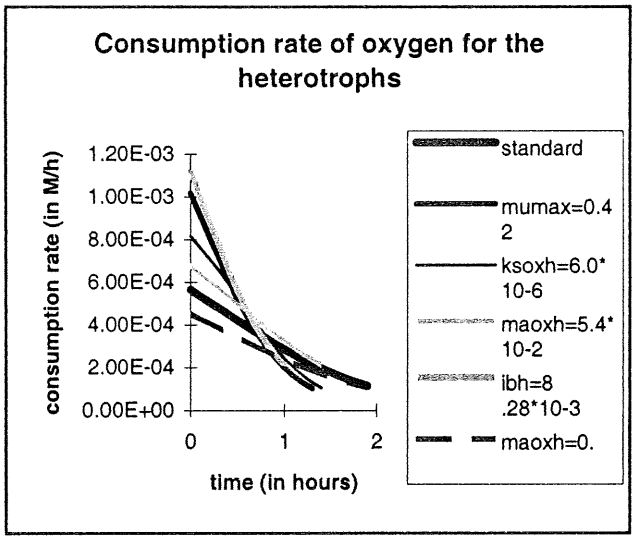


Figure 47: The consumption rate of oxygen was plotted for the heterotrophs

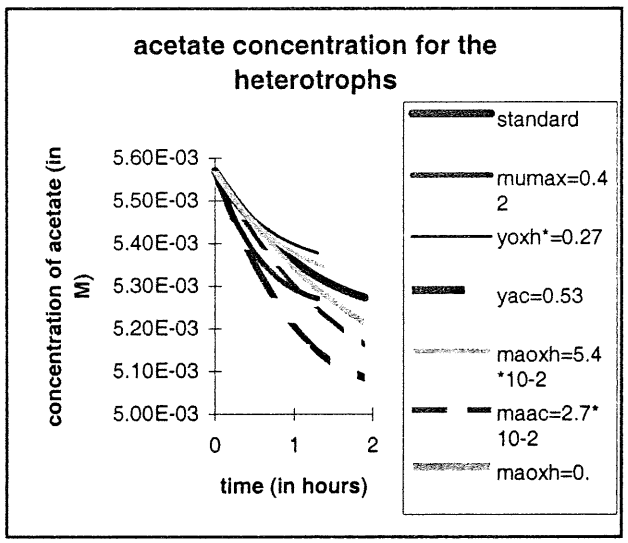


Figure 48: The concentration of acetate was plotted for the heterotrophs

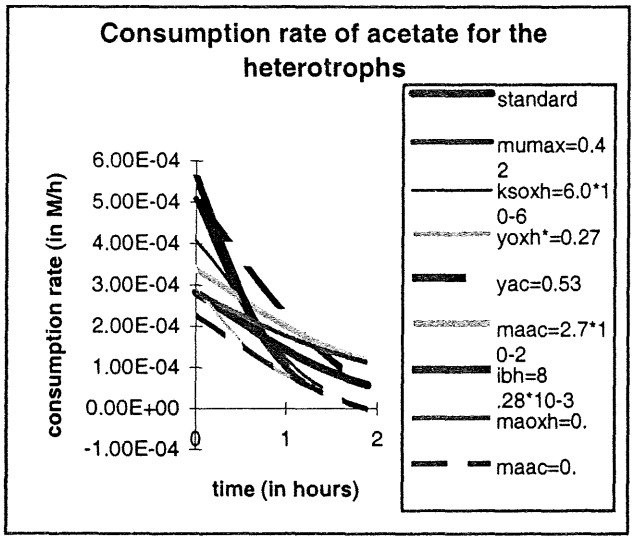


Figure 49: The consumption rate of acetate was plotted for the heterotrophs

Some differences between the model analysis of the methanotrophs and the heterotrophs is the greater influence of changing the biomass of the heterotrophs on the consumption rates of the substrates. An explanation for this difference could be the higher initial amount of biomass of the heterotrophs. An other difference is the influence on the results by changing the maintenance coefficients of oxygen and acetate. The initial values are also higher than for the methanotrophs and it seems that at higher initial values these parameters have more influence.

From this chapter it can concluded that the time period for growth is shorter for the model than for the batch experiment, due to the high consumption rates of oxygen. Therefore the concentration of oxygen in the model soon reaches zero.
An explanation for the high consumption rate of oxygen could be that the initial amount of biomass in the model has been calculated too high in the model in comparison to the batch experiment. From the analysis of the model with a set of parameters from literature it can be seen that when the biomass of the bacteria increases, it has a great impact on the consumption rates of the different substrates. However the problem could not be solved with changing only this parameter. Changing the maximum relative growth rate has also a great influence on the increase of the consumption rate as has the half saturation constant at lower concentrations of the substrate. Also the yield factors and the maintenance coefficients for oxygen for the heterotrophs have influence.
An other difference between the model results and the results of the experiment is the growth of the biomass, that occurs in the batch experiment model just after 50 hours. An explanation for the absence of growth could be a lag phase of the bacteria. At that time no growth occurred, however there is still consumption of the substrates for the maintenance of their cells. Due to bad circumstances the bacteria increase their maintenance and when the circumstances are good again growth can occur.
From the models with parameters from the batch experiment it seems that oxygen is the limiting substrate as was in the batch experiment. However in the model with parameters from the literature it has been found that methane is the limiting substrate. This was due to the lower

concentrations of methane.
From this it can be concluded that it is important to make a good estimation of the kinetic parameters, because of the influence the parameters have on the results of the biomass.

Chapter 5 Conceptual ideas for the extension of the kinetic model to a rhizosphere model

The final aim is to make a model of the competition for oxygen between the methanotrophs and heterotrophs in the environment of a single rice root.
As a start up an overview is given of literature for the most important equations and extra parameters needed for such a model.

The aim of the model is to make a more realistic estimation of the contribution of the methanotrophs to the oxidation of methane by taking diffusion and production processes into account. This could be done by taking into account the rhizosphere of a single rice root in the soil. The rice root could be assumed to be a plate, for the calculation of diffusion processes in an easy way. Besides not much was known about the diffusion processes in a rice root. Another assumption that should be made is that the concentration of oxygen is zero at a distance of 3 mm (length of the rhizosphere) from the rice root and that the concentration of methane should be saturating at that distance from the root. The length of the rhizosphere depends on the diffusion coefficients of the substrates and on the density of the roots in the rice soil.
The following processes should have to be taking into account in the model:

1) Diffusion equations of the substrates oxygen, methane and acetate in mol/m^2 soil*day. The concentration of the substrates in time are dependent on a rate (transpiration rate or conductive water flow), a diffusion coefficient and on the concentration in the previous layer. The soil should be divided into small layers for the calculation of the diffusion of the substrates. Only a diffusion rate for oxygen in the rice root has been found, which can be taken as a condition for the maximum value for the diffusion rate of oxygen. This value has been found by Armstrong (1971). The rate of oxygen diffusion from the root surface of paddy rice root was 0.135 to 0.153 mol O_2 *day (non-waterlogged) and 0.18 to 0.21 mol O_2 *day (water-logged) at 23 °C. An explanation was that the gas volume ratio is higher in a plant, which has to endure a wet condition than in one which has not. It is also higher in a plant grown in a paddy than in one grown in an upland condition (Kumazawa et al., 1984).

2) Production processes for oxygen and acetate should have been taken into account. It should be assumed that there is no production of methane in the presence of oxygen. For the production of oxygen the root oxygen release could be calculated. First some data are given on the mechanism of oxygen release and some values of the methane oxidation potentials. Thereafter two methods for the calculation of the root oxygen release are presented.
In some marsh plants the root oxidising activity is nine times greater than can be accounted for by its oxygen diffusion. Therefore it is considered that enzymatic oxidation is the principal mechanism for this oxidising activity (Armstrong, 1967). This is however not the case for rice. The source of oxidising power of paddy rice roots has been thought to be the hydrogen peroxide produced by the roots. It is considered that in normal growth conditions the oxidising power of the root is located where the hydrogen peroxide is generated (Kumazawa, 1984).
The rice root consumes oxygen by aerobic respiration just like the roots of common upland crops. The oxygen required for this aerobic respiration is not supplied from outside the root, but is transported from the foliar parts of the plant to the root. The gas contained in the root of the rice seedlings was 2.1 to 14.0 % oxygen (Raalte, 1940). Even if oxygen in the medium in which the roots were immersed was consumed by oxygen-consuming micro-organisms, the oxygen content in the gas mixture of the roots was still 3 % (Kumazawa, 1984). Although

plants transported large amounts of oxygen between shoots and roots, most of that oxygen is consumed before it reaches the bulk solution. The oxygen content of solutions bathing the root systems typically remained at or below 31 M. Rates of oxygen generation (by root oxygen release) within the root zone solutions were generally well below 3.50*10 mol O_2 /plant*day for most experimental plants (Bedford et al., 1991).
The root oxygen release can be calculated in two ways.

1) By calculation from different processes. From the concentration of oxygen in the atmosphere and the resistances in the root the diffusion of oxygen from the root into the rhizosphere is calculated (models by Armstrong, 1971). Plant transport into the root environment was governed by soil redox capacity. Studies of ROL in plants must therefore consider soil O_2 demand, which strongly governs the quantity of O_2 transport through wetland plants into the soil environment (Kludze et al., 1995). This is important as the concentration in the soil influences the diffusion.

2) From data of a table with diurnal variation in the root oxygen release for different growth stages in terms of α-naphythalamine oxidation calculated into µmol O_2/(g dry weight of roots*day) with the following formula:

µmol α-naphythalamine / (g dry root*day)) * (x*y/z)

x = stoichiometric factor (3/2)
y = the fraction of the day that has been elapsed
z = the molair mass of α-naphythalamine in g/mol (143)

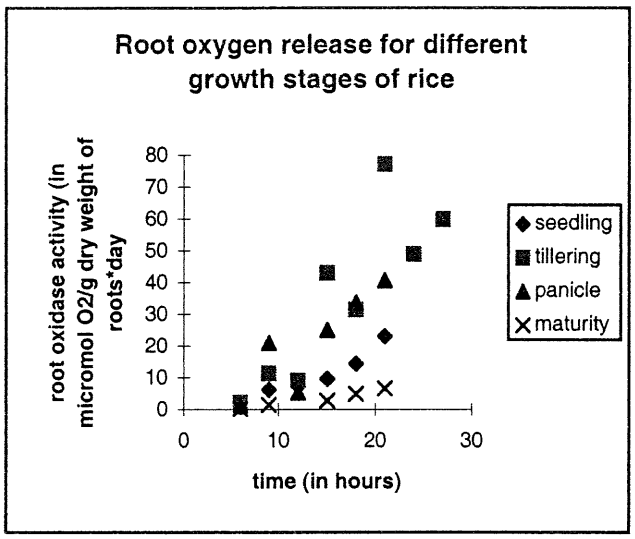


Figure 50: Root oxidase activity of the root tip in µmol O_2/(g dry weight of roots*day) for the different growth stages of rice (Satpathy et al., 1997)

The figure shows that root oxygen release depends on the time in the growing season. Besides the root oxygen release it shows also a distinct diurnal pattern. The root tip exhibited higher oxidase activity (reaching a minimum between 12.00 and 15.00 and increased thereafter reaching the maximum around midnight) at all the four rice growth stages.
Methane emission shows also a distinct diurnal pattern. Especially at tillering, panicle

initiation and maturity stages of a field-grown rice crop, with maximum emission in the early afternoon (12.00 to 15.00) followed by a decline to a minimum around midnight (Satpathy et al., 1997). This can be a direct influence of the root oxygen release on methane emission via rhizospheric methane oxidation.

The production of acetate depends on the exudation flux in mol/m^2*day, the rate of soil mineralisation and on root decay. Root exudation and root decay take place in the rhizosphere and soil mineralisation takes place in both rhizosphere as the bulk soil. Soil mineralisation is the release of low organic compounds by the breakdown of soil organic matter. Soil mineralisation is not constant, but decreases in time due to a decrease in easily accessible organic material. Very few data exist on root exudation. It seemed that root exudation reaches a maximum between 50 to 70 days and then decreases again. The release of organic matter from roots is the result of three processes root exudation, root mortality and root decay. All depend on the time in the growing season.

From the model about competition between pure cultures of methanotrophs and heterotrophs it appears that the number of bacteria present is of influence on the impact on the consumption of especially oxygen and therefore on the quantity of oxidation of methane by methanotrophs. It was already known that methanotrophs colonised the rice roots (Kimura et al., 1988). Here the methanotrophs do not compete with the heterotrophs for oxygen. The heterotrophs are not present in the roots because no free acetate is available. The colonisation is usually more active in the region of lateral root emergence than in other regions except the region with a large number of root hairs and mucigel-like substance (Kimura et al., 1988). A significantly increased number of MOB (Methane oxidising bacteria) has been found only in soil immediately around the roots ($1.2*10^{-6}$ g/g fresh weight), corresponding to a layer of 0.1 to 0.2 mm. The average number of MOB in homogenised roots is larger than on the rhizoplane and increased with plant age (Bosse et al., 1997). MOB are also found in surface-sterilised roots and basal culms, indicating the ability of these bacteria to colonise the interior of roots and culms (Bosse et al., 1997). The number of total root MOB after homogenisation increases much more with plant age than does the rhizoplane population, indicating that growth conditions are better in the roots as a whole than on their surface.
Figure 51 shows the presentation of a rhizosphere of a single rice root with the diffusion and production processes of the substrates taken into account.

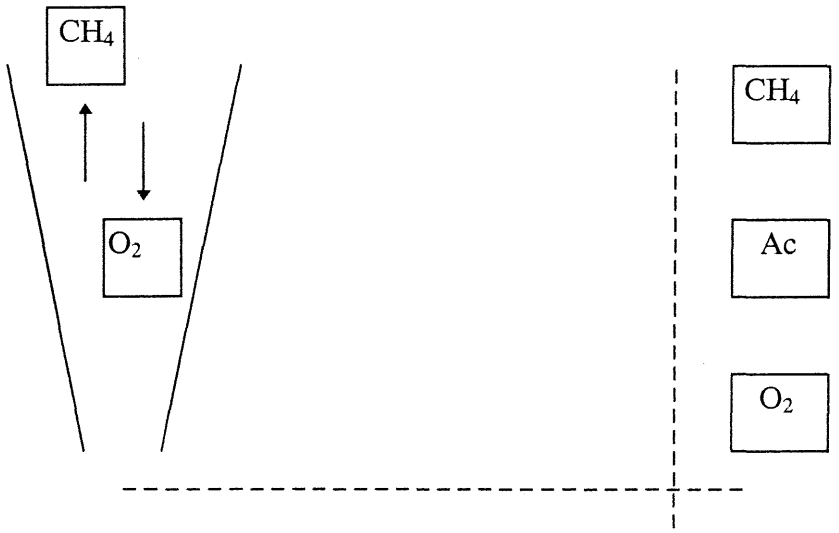


Figure 51: The rhizosphere of a single rice root

Chapter 6 General discussion

6.1 Discussion

In this study experiments of pure cultures of methanotrophs and heterotrophs have been performed and a competition model for oxygen between these bacteria in the rhizosphere has been made. First the results of the batch experiment and the parameters found in literature are discussed and thereafter the results of the batch experiment and the results of the model.
The half saturation constant for the different substrates in the batch experiment were quite similar to the ones found in the literature, taking the standard errors into account. However the half saturation constant for oxygen for the methanotrophs which was found in the batch experiment was lower than for the heterotrophs and half saturation constant found in the literature was higher than for the methanotrophs. If the ratio of the concentration and the half saturation constant for a substrate is very low, due to low concentrations and a high half saturation constant, then the relative growth rate becomes very low. In the model analysis this parameter has been changed to measure its influence on the results.
In the experiment the maximum relative growth rate for the methanotrophs was set at 0.08 h^{-1} and the maximum relative growth rate of the heterotrophs calculated was 0.32 h^{-1} with oxygen limitation and 0.05 h^{-1} with acetate limitation. In literature the same range of values for the heterotrophs have been found. But the difference in the maximum relative growth rate for both types of limitation has to be very small, because the maximum relative growth rate is independent on the type of substrate. Besides not much difference has been seen between the figures with relative growth rates for both types of limitations for the heterotrophs. Also the maximum relative growth rate with oxygen limitation is much higher than the relative growth rates found in the experiment. Those high standard errors for the calculated parameters in the experiment have to be taken into account. Another control for the calculation of the kinetic parameters have been the BOM experiments. A maximum relative growth rate which is five times lower than in the batch experiment has been calculated for the heterotrophs. Therefore more evidence was found for the lower value of the maximum relative growth rate for the heterotrophs.

There have been some problems in describing the experiment with a model. In the batch experiment hardly any consumption of the substrate after 50 hours has been found. However still growth occurs after 50 hours. Compared to the model the growth period was very short, because the concentration of oxygen has become limiting. An explanation for the difference in the time period for growth can be the lower consumption rates of oxygen found in the experiment. The consumption rates depend on the growth of the bacterium, the inverse of the yield factor, the maintenance coefficient and the amount of biomass of the bacterium. The maintenance coefficient and the yield factors have not been calculated in the experiment and are based upon literature. Literature (Panikov, 1995) shows that the bacteria could decrease their maintenance if the concentrations of the substrate have become very low as was the case in the experiment. Maybe the value of this coefficient in the model was chosen too high.
The maximum relative growth rates are also based upon literature, due to the high standard errors. The number of bacteria have been calculated from optical density measurements with a calibration curve and the amounts of bacteria are based upon an assumption. When the biomass increases with a factor ten, based upon a different value found for the assumption, the consumption rate increased

also with a factor ten. It appeared that the consumption rate of oxygen increased with an increase of the biomass with the same value as for the maximum relative growth rate and the yield factor. Another explanation can be the low activities of the bacteria for oxygen. Maybe the bacteria in the batch experiment needed to adapt first to the new circumstances before the consumption of substrates could start. Due to these low activities the consumption rate could be also very low. Decreasing the half saturation constant for oxygen for both bacteria increased the consumption rate of oxygen also. At lower concentrations of oxygen the half saturation constant has more influence on the increase of the consumption rate than the maximum relative growth rate and the yield factor.
An explanation for the absence of growth can be the occurrence of a lag phase of the bacteria. No lag phase was accounted for in the model. The consumption in the first 50 hours can be due to an increase of the maintenance coefficient and a decrease of the yield factor in absence of growth. Then the consumption rate would become also higher, which was already very high.

It seems that in the batch experiment the concentration of oxygen became too low for growth in all the batches (beneath the half saturation constant) and therefore oxygen should be the limiting substrate. If the parameters of the batch experiment have been used as input for the model it has also been found that oxygen is the limiting substrate. From the model with input parameters from literature it was found that methane was the limiting substrate. To know which bacterium will survive in the rhizosphere, concentrations of the substrates found in the rhizosphere were used in the analysis of the model with parameters from literature. From this it can be seen that methane was the limiting substrate rather than oxygen. If methane is the limiting substrate then the methanotrophs could not compete with the heterotrophs for oxygen.
Literature (Bosse et al., 1997) shows that methane could not be the limiting factor in soil, because the threshold concentration for methane was much lower than the concentrations of methane in the soil. Besides not the whole population in the soil seemed to be active, indicating that oxygen is limiting again. Maybe methane can be limiting in the roots because in the roots the threshold concentration for methane is higher than in the soil and the methane concentrations were lower.
Literature also shows that methane oxidation potentials measured at 30 μM methane (far below the concentration used in the model) were much lower in soil than in roots. From this it can concluded that this is enough methane in the roots for oxidation. Because more bacteria were active in the roots more methane might be oxidised, if enough methane is present in the roots. It seems that the roots are the best place for oxidation. In the roots more oxygen and less methane is present than in the soil. For the oxidation more oxygen is needed than methane and besides in the roots the methanotrophs do not have to compete with the heterotrophs for oxygen.
The methane oxidation in the roots should also be higher than in the rhizosphere, if more bacteria are present, a greater part of methanotrophs is active and if the concentration of methane is not limiting. It is already known that the bacteria are also present in the roots. The average number of MOB (Methane Oxidising Bacteria) in homogenised roots was larger than on the rhizoplane and increased with plant age. The number of total root MOB increased also much more than did the rhizoplane population, indicating that the growth conditions were better in the roots as a whole than on their surface (Bosse et al., 1997).

6.2 Conclusions

To estimate the outcome of the competition for oxygen between the methanotrophs and the heterotrophs the kinetic parameters, the concentration of the substrates and the place of the bacteria are important. The first aim of this thesis was to estimate the kinetic parameters. The estimated kinetic parameters from experiment were not significant. The half saturation constants are in the same range, when the standard errors are taken into account. Also much variation exists of the parameters as can been seen in the literature review (§ 4.3).
Increasing the maximum relative growth rate has more influence on the growth of the bacteria than decreasing the half saturation constant with the same value. Therefore a good estimation of the value of the maximum relative growth rate is needed for the estimation of the growth of both bacteria. Also the amount of biomass and the yield factor have much influence on the relative growth rate of the bacteria. A good estimation of the half saturation constant for oxygen is important for the consumption rates of the substrates especially at low concentrations. The maximum relative growth rate, the amount of biomass, the yield factor and the maintenance coefficient have also influence on the consumption rates of the substrates. Therefore a good estimation of these parameters is very important for the outcome of the competition for oxygen between the methanotrophs and the heterotrophs.

From the experiments and model with a set of parameters from the batch experiment it can be concluded that oxygen is the limiting substrate when based on half saturation constants for pure cultures and for the concentrations used from the batch experiment. From the competition model using saturating concentrations, it can be seen that the methanotrophs couldn't compete with the heterotrophs in the rhizosphere because of worse intrinsic characters for growth and methane limitation. This was also the case when concentrations in the rhizosphere have been used. However if methanotrophs are present in the roots, they don't have to compete for oxygen with the heterotrophs, because no heterotrophs are available in the roots.
More methane oxidation might take place in the roots, because of the potential higher rates here than in the rhizosphere. To determine quantitatively the influence of the methanotrophs on the oxidation of methane in a rice paddy the production and diffusion processes have to be taken into account for the competition for oxygen between those two bacteria. This should be done in a following study.

Important parameters for the competition between the methanotrophs and the heterotrophs and the impact of the methanotrophs on the oxidation of methane are:

- the maximum relative growth rate
- the half saturation constant
- the yield factor
- the maintenance coefficients
- the amounts of biomass
- the concentrations of the substrates
- the living-place of the bacteria

6.3 Recommendations

- More distinction in the relative growth rates for different concentrations of the substrate. This can be done by introducing larger variations of the concentrations, or by BOM experiments where it is assumed that the biomass is constant. Maximum relative growth rate then can be calculated by the specific activities and the yield factors.
- Measuring the concentrations of the substrates in a different way, for example with an oxygen electrode as has been done in the BOM experiment. The carbon substrate concentrations can be calculated with stoichiometry.
- Experiments can be done about the competition between both bacteria, using soil from the rhizosphere. These results then can be compared with the results of the competition model.
- Chemostat experiments can be done for measuring the growth kinetic parameters for mono-cultures of the bacteria, or the competition can be measured with a mixed culture.
- Estimations of the amount of biomass in the roots, the rhizosphere and in the soil can be done by the Most Probable Number method. A good estimation of the biomass seems important for the growth of the bacteria and the consumption rates of the substrates.
- An extension of the competition model which takes diffusion and production processes into account should be made. For a first estimation input parameters from literature for a single rice root could be used. This model should be a better approximation of the field situation than the kinetic model alone.

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Appendix A Calibration curves of oxygen, methane and acetate

Calibration curves of the substrates have been made to convert the areas measured with a chromatograph into a number of moles or a concentration of the substrate.

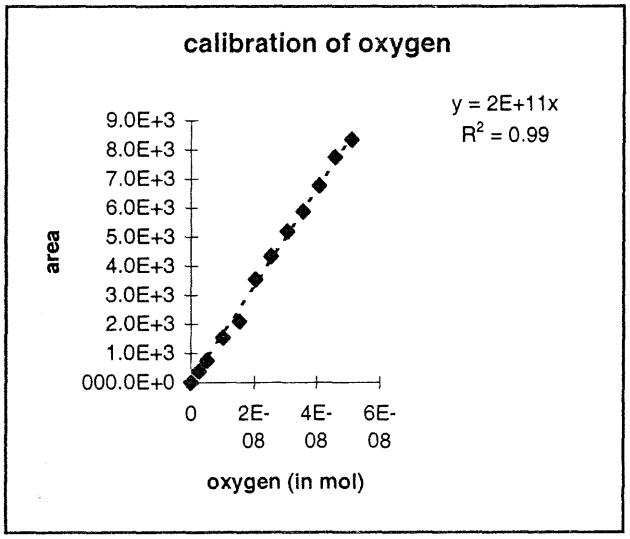


Figure 52: Number of moles of oxygen plotted against areas measured

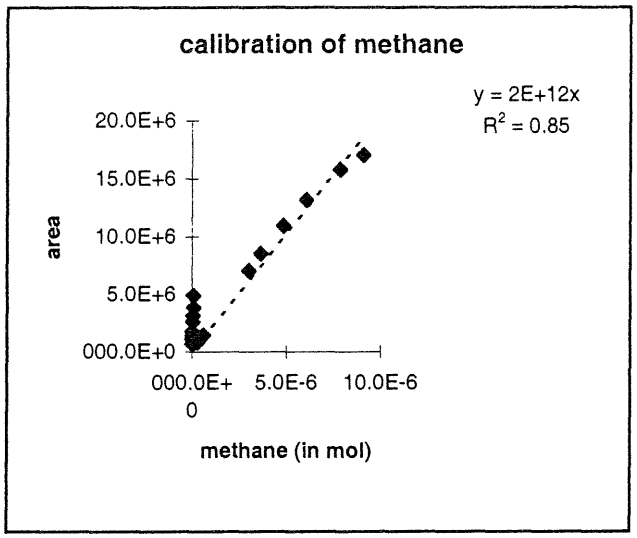


Figure 53: Number of moles of methane plotted against areas measured

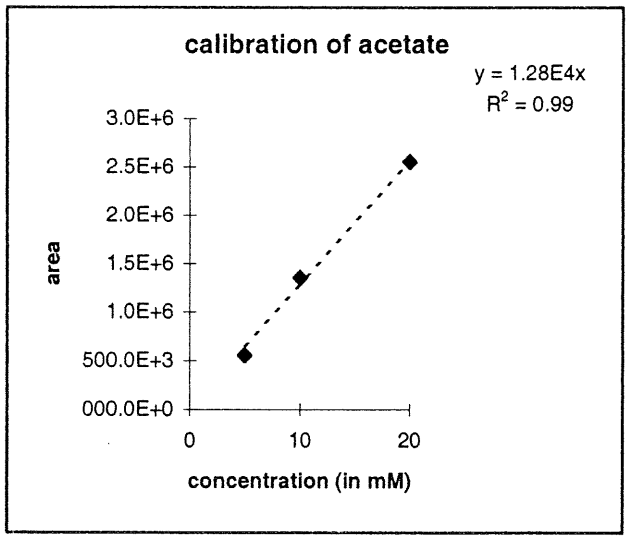


Figure 54: Concentraton of acetate plotted against areas measured

Appendix B Hofstee and Lineweaver Burk plots

There are several methods for the estimation of the half saturation constant and the maximum relative growth rate. Those methods transform the Monod equation in linear equations so that the parameters can be easily estimated from the figures.
Two methods which have been used in paragraph 2.4 are described below.

Hofstee method

The Hofstee method transforms the Monod equation

$\mu = \mu max*(S / (S+Ks))$
into
$\mu = \mu max - Km*(\mu / S)$

When the relative growth rate divided by the concentration of the substrate is plotted against the relative growth rate rate, the maximum relative growth rate can be calculated at the intersection with the y-axis and the half saturation constant as the negative slope.

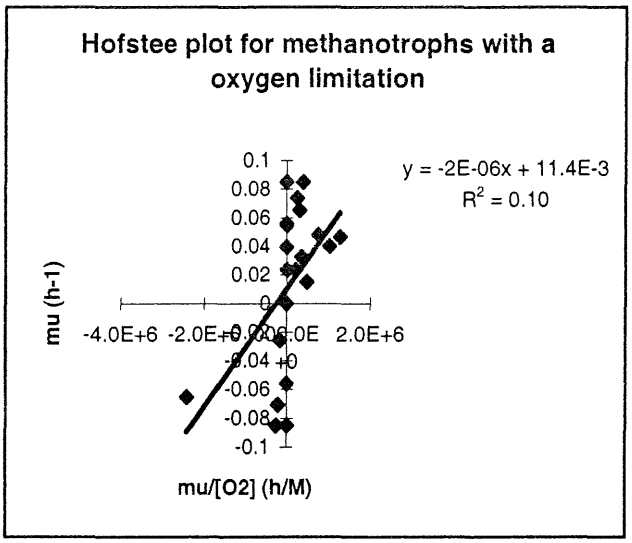


Figure 55: The relative growth rate divided by the concentration of oxygen plotted against the relative growth rate for the methanotrophs

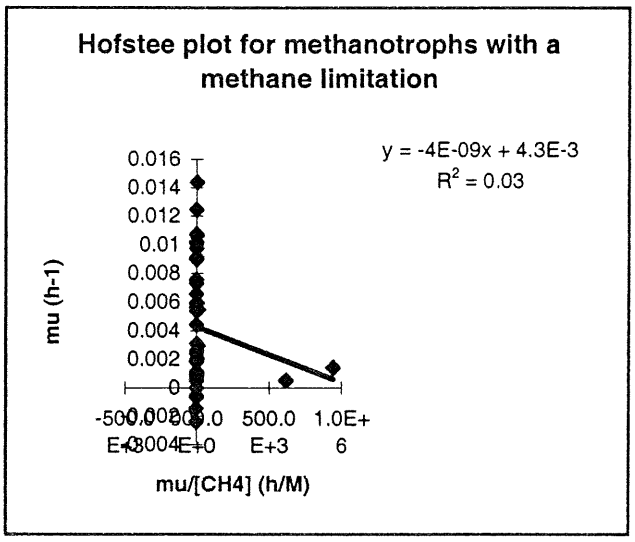


Figure 56: The relative growth rate divided by the concentration of methane plotted against the relative growth rate for the methanotrophs

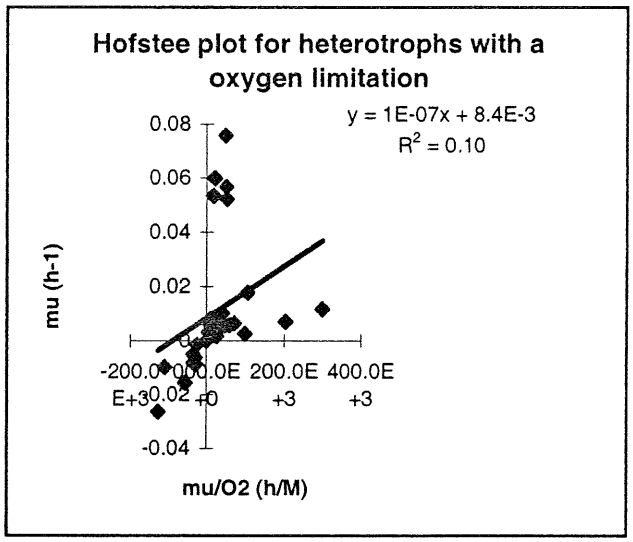


Figure 57: The relative growth rate divided by the concentration of oxygen plotted against the relative growth rate for the heterotrophs

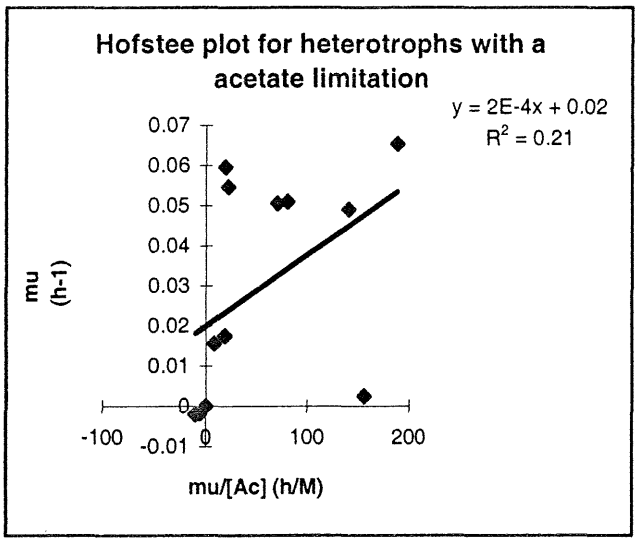


Figure 58: The relative growth rate divided by the concentration of acetate plotted against the relative growth rate for the heterotrophs.

These figures could not be used for the estimation of the half saturation constant and the maximum relative growth rate, due to clustering of the data. This is caused by the fact that not much variation exists in the relative growth rate for the different concentrations of the substrate. Also negative relative growth rates have been found, caused by the absence of growth at the start of the experiment.

Lineweaver and Burk method

The Lineweaver and Burk method transforms the Monod equation into

$1/\mu = (Ks / \mu max)*1/S + 1/\mu max$

When the inverse of the concentration of the substrate is plotted against the inverse of the relative growth rate, the inverse of the maximum relative growth rate can be calculated as the slope and the negative inverse of the half saturation constant as the intersection with the x-axis.

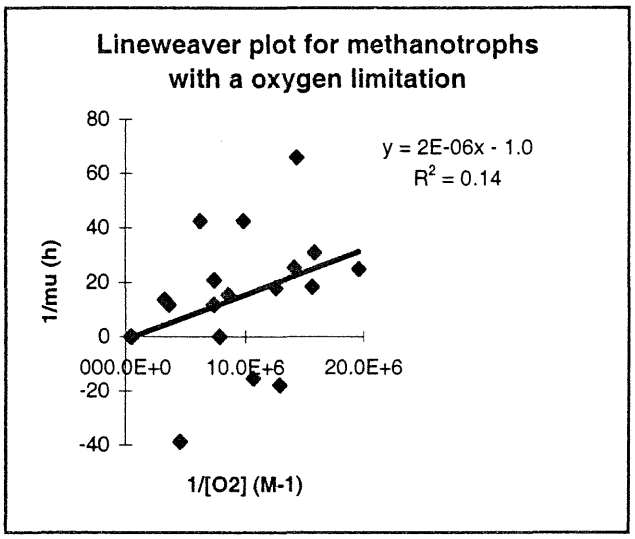


Figure 59: The inverse of the concentration of oxygen plotted against the inverse of the relative growth rate for the methanotrophs

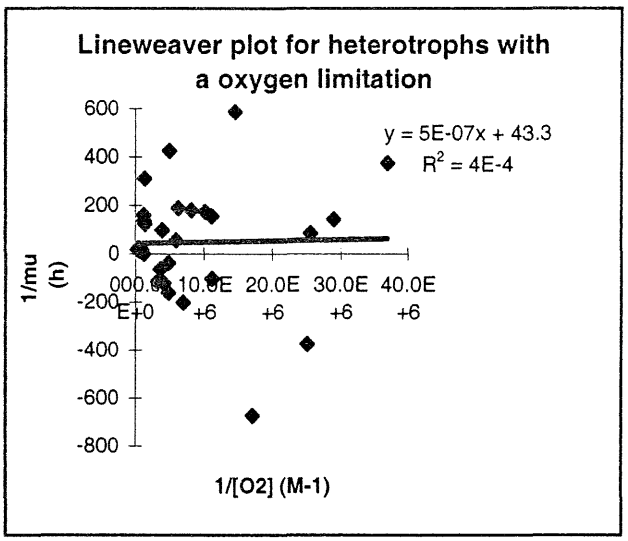


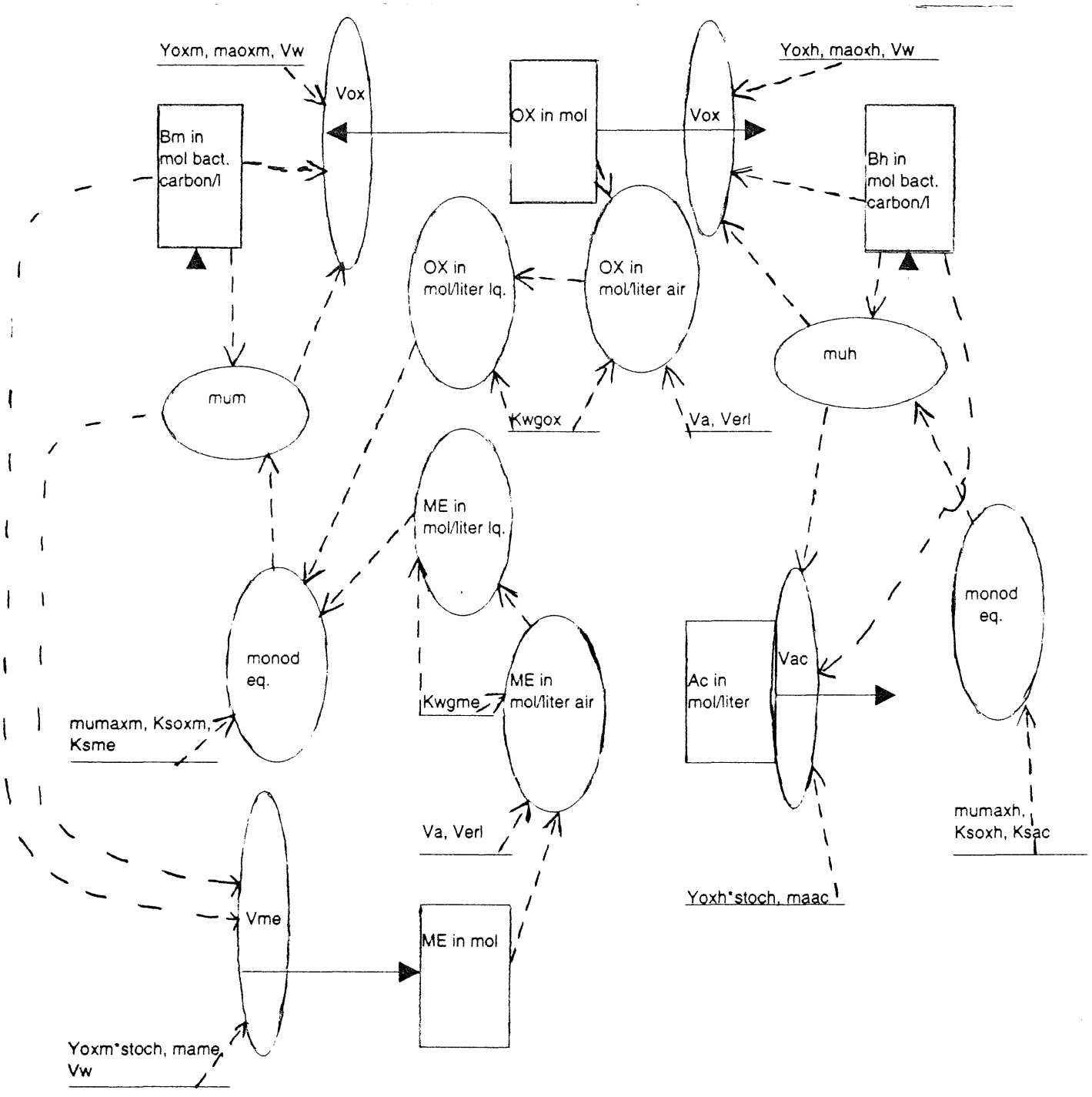
Figure 60: The inverse of the concentration of oxygen plotted against the inverse of the relative growth rate for the heterotrophs

These figures could not be used either. For the methanotrophs with oxygen limitation the estimation of the kinetic parameters is very different from the calculated values with the linear optimalisation program and values found in the literature. For the heterotrophs with oxygen limitation the data vary too much for fitting a line. Therefore the coefficient for determination is very low, as is the case for the methanotrophs with oxygen limitation..

Appendix C Model presentation

This Appendix shows a Forrester diagram of the competition model for oxygen between the methanotrophs and the heterotrophs for the circumstances in the batch experiment. Also the set-up of the model with its differential equations and dimensions is given with and without competition of the bacteria. The model without competition is equal for both bacteria, except that for each model one bacteria does not grow. For example in the model for the heterotrophs, the biomass of the methanotrophs and the concentration of methane have been set at zero.

Forrester diagram:



```
ITLE Competition model between bacteria for oxygen with a set of...
     parameters from literature
NITIAL
 Data input
NCON      IBM=3.97E-3; IBH=4.14E-3; ITAMGO=3.62E-5; IME=0.165E-3;...
          IAC=1.5E-3; IC=0.
ONSTANT   STOCH=2.; FACT1=1.; FACT2=2.; KWGOX=0.03; VA1=3E-6;...
          VW1=5.28E-2
'ARAMETER MUMAXM=0.08; MUMAXH=0.21; KSOXM=4.7E-6;...
          KSME=2.5E-6 ;KSOXH=1.38E-6; KSAC=1.56E-3;...
          YOXM=0.24; YOXH=0.53;...
          MAOXM=1.74E-3; MAOXH=2.7E-2;...
          MAME=8.7E-4; MAAC=1.35E-2
 Data output
'RINT     BM, BH, NBM, NBH, CONSOX, CONSME, CONSAC,...
          MUM, MUH, NC, ME, CWO, AC
 The independent variable time
'IMER     STTIME=0.0; DELT=0.001; PRDEL=0.01; FINTIM=100.
 The integration method
'RANSLATION_GENERAL DRIVER='RKDRIV'

)YNAMIC
 State  variables
3M=INTGRL (IBM ,NBM)
3H=INTGRL (IBH ,NBH)

'AMGO=INTGRL (ITAMGO ,RAMGO)
'AO=TAMGO / (VA1 + KWGOX*VW1)
'WO=KWGOX*CAO

IE=INTGRL (IME, NME)
\C=INTGRL (IAC ,NAC)
C= INTGRL (IC  ,NCARB)

 Rate variables
NBM=GROWM
NBH=GROWH

RAMGO=-CONSOX*VW1
NME=-CONSME
NAC=-CONSAC
NCARB=((GROWM*(1.-YOXM*STOCH))/YOXM*STOCH + MAME*FACT1*BM) + ...
      ((GROWH*(1.-YOXH))/YOXH + MAAC*FACT2*BH)

GROWM=MUM*BM
MUM=MUMAXM*(CWO/(KSOXM+CWO))*(ME/(KSME+ME))
GROWH=MUH*BH
MUH=MUMAXH*(CWO/(KSOXH+CWO))*(AC/(KSAC+AC))

CONSOX=(GROWM/YOXM) + (MAOXM*BM) + (GROWH/YOXH) + (MAOXH*BH)
CONSME=(GROWM/(YOXM*STOCH)) + (MAME*BM)
CONSAC=(GROWH/(YOXH*STOCH)) + (MAAC*BH)

NC=(IME*FACT1 + IAC*FACT2 + IBM + IBH -...
  ME*FACT1 - AC*FACT2 - BM - BH - C)/...
```

```
   (IME*FACT1 + IAC*FACT2 + IBM + IBH)

FINISH CWO<0.
FINISH ME<0.
FINISH AC<0.
END
STOP
```

```
'ITLE Competition model between bacteria for oxygen with a set of...
      parameters from the batch experiments for the heterotrophs
 NITIAL
  Data input
 NCON      IBM=0.0; IBH=4.14E-3; ITAMGO=1.54E-5; ITAMGM=0.0;...
           IAC=5.57E-3; IC=0.
 ONSTANT   STOCH=2.; FACT1=1.; FACT2=2. ; VA=0.094; VW=0.026;...
           KWGOX=0.03; KWGME=0.03
 ARAMETER MUMAXM=0.08; MUMAXH=0.21; KSOXM=3.45E-6;...
           KSME=9.5E-5;KSOXH=1.2E-5; KSAC=2.56E-4;...
           YOXM=0.24; YOXH=0.53;...
           MAOXM=1.74E-3; MAOXH=2.7E-2;...
           MAME=8.7E-4; MAAC=1.35E-2
  Data output
 RINT       BH, CWO, AC, NBH, CONSOX, CONSAC,...
            MUH, NC, C, TAMGO
  The independent variable time
 IMER      STTIME=0.0; DELT=0.001; PRDEL=0.1 ;FINTIM=100.
  The integration method
 RANSLATION_GENERAL DRIVER='RKDRIV'

 YNAMIC
  State  variables
 BM=INTGRL (IBM ,NBM)
 BH=INTGRL (IBH ,NBH)

 TAMGO=INTGRL (ITAMGO, RAMGO)
 CAO=TAMGO/(VA + KWGOX*VW)
 CWO=KWGOX*CAO

 TAMGM=INTGRL (ITAMGM, RAMGM)
 CAM=TAMGM/(VA + KWGME*VW)
 CWM=KWGME*CAM

 AC=INTGRL (IAC ,NAC)
 C=INTGRL (IC, NCARB)


* Rate variables
 NBM=GROWM
 NBH=GROWH

 RAMGO=-CONSOX*VW
 RAMGM=-CONSME*VW
 NAC=-CONSAC
 NCARB= ((GROWM*(1.-YOXM*STOCH))/YOXM*STOCH + MAME*BM) +...
        ((GROWH*(1.-YOXH))/YOXH + MAAC*FACT2*BH)

 GROWM=MUM*BM
 MUM=MUMAXM*(CWO/(KSOXM+CWO))*(CWM/(KSME+CWM))
 GROWH=MUH*BH
 MUH=MUMAXH*(CWO/(KSOXH+CWO))*(AC/(KSAC+AC))


 CONSOX=(GROWM/YOXM) + (MAOXM*BM) + (GROWH/YOXH) +(MAOXH*BH)
```

```
* O2 is de enige electron acceptor en dus geen correctie ten opzichte
* het totaal aan electron acceptoren
CONSME=(GROWM/(YOXM*STOCH)  +(MAME*BM))
CONSAC=(GROWH/(YOXH*STOCH) +(MAAC*BH))

NC= ((ITAMGM/(VA + VW))*FACT1 + IAC*FACT2 + IBM + IBH - ...
    (ITAMGM/(VA + VW))*FACT1 - AC*FACT2 - BM - BH - C)/...
    ((ITAMGM/(VA + VW))*FACT1 + IAC*FACT2 + IBM + IBH)

FINISH CWO<0.
FINISH CWM<0.
FINISH AC<0.
END


*Dimensions of the parameters:

* INITIAL:
* BM is the concentration of the methanotrophs in
* (mol bacterial carbon/liter)
* BH is the concentration of the heterotrophs in
* (mol bacterial carbon/liter)
* TAMGO AND TAMGME are the amounts of oxygen and methane
* in the gas phase in (mol)
* ME is the concentration of methane in (mol CH4/liter)
* AC is the concentration of acetate in (mol Ac/liter)
* C is the concentration of carbon respirated by the methanotrophs
* and the heterotrophs in (mol carbon/liter)
* MUMAXM is the maximum relative growth rate of the methanotrophs
* in (h-1)
* MUMAXH is the maximum relative growth rate of the heterotrophs
* in (h-1)
* KSOXM is the half saturation constant of the methanotrophs for oxygen
* in (mol O2/liter)
* KSME is the half saturation constant of the methanotrophs for methane
* in (mol CH4/liter)
* KSOXH is the half saturation constant of the heterotrophs for oxygen
* in (mol O2/liter)
* KSAC is the half saturation constant of the heterotrophs for acetate
* in (mol Ac/liter)
* YOXM is the yield factor of methanotrophs for oxygen in
* (mol bacterial carbon/mol substrate)
* YOXH is the yield factor of heterotrophs for oxygen in
* (mol bacterial carbon/mol substrate)
* MAOXM is the maintenance cofficint of the methanotrophs for oxygen
* in (mol substrate/(mol bacterial carbon*h))
* MAOXH is the maintenance cofficint of the heterotrophs for oxygen
* in (mol substrate/(mol bacterial carbon*h))
* STOCH is a stochastic factor and used for the transposition of
* the yield factor for oxygen in the yield factor for methane or acetate
* in (mol O2/mol (CH4 or Ac))
* FACT1 is the ratio of the number of moles of carbon in methane
* and the number of moles of methane in (mol carbon/mol CH4)
* FACT2 is the ratio of the number of moles of carbon in acetate
* and the number of moles of acetate in (mol carbon/mol Ac)
```

```
* VA is the volume of the gas phase in the batch in (liter)
* VW is the volume of the water phase in the batch in (liter)
* VA1 is the volume of the gas phase in the root in (liter)
* VW1 is the volume of the water phase in the rhizosphere in (liter)
* KWGOX is the ratio of the concentration of oxygen in the gas phase
* and in the liquid phase in ((mol/liter gas)/(mol/liter liquid))
* KWGME is the ratio of the concentration of methane in the gas phase
* and in the liquid phase in ((mol/liter gas)/(mol/liter liquid))


* DYNAMIC:
* NBM and GROWM  are the net growth rates of the methanotrophs in
* (mol bacterial carbon/(liter*h))
* NBH and GROWH are the net growth rates of the heterotrophs in
* (mol bacterial carbon/(liter*h))
* MUM is the relative growth rate of the methanotrophs in (h-1)
* MUH is the relative growth rate of the heterotrophs in (h-1)
* CAO and CAM are the concentrations of oxygen or methane in
* the gas phase in (mol/liter)
* CWO and CWM are the concentrations of oxygen or methane in
* the liquid phase in (mol/liter)
* RAMGO and RAMGM are the net rates of oxygen or methane in
* (mol/h)
* CONSOX, CONSME and CONSAC are the consumption rates of oxygen,
* methane and acetate in (mol/(liter*h))
* NAC is the net rate of acetate in (mol Ac/(liter*h))
* NCARB is the rate of respiration of carbon by the methanotrophs
* and the heterotrophs in in (mol carbon/(liter*h))
* NC is the ratio of the net concentration of carbon and
* the initial concentration of carbon in (mol carbon/liter)
```

Appendix D The different definitions of the maintenance coefficient

Schatten van het verlies van bacteriele biomassa

6-4-1998

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Er zijn twee parameters die de bacteriele biomassa verliezen beschrijven. Ten eerste is dat een relatieve sterfte snelheid en ten tweede is dat de maintenance. Relatieve sterfte snelheid is gedefinieerd als de snelheid van sterfte van bacteriën gedeeld door het aantal aanwezige bacteriën. Maintenance daarentegen is gedefinieerd als de energie die wordt geconsumeerd door bacteriën voor andere functies dan de produktie van nieuw celmateriaal om het organisme gezond te houden. De biomassa gaat bij dit laatste proces niet direct achteruit. Er zijn verschillende manieren om met deze biomassa verliezen om te gaan. Ik zal vier methoden beschrijven

a) constant relatief verlies van biomass snelheid

De meest eenvoudige benadering, die waarschijnlijk ook het meest praktisch toepasbaar is, is het beschouwen van een constant relatief verlies aan biomassasnelheid. Opvallend is dat in de gereviewde literatuur niemand beide processen van biomassa verlies apart beschouwt.

De eerste groep beschouwt enkel de relatieve sterfte, D met als eenheid day^{-1}. Er wordt dan geen aparte maintenance meegenomen, noch in de biomassa veranderingen, noch in de substraat veranderingen. Dit levert (Lovley and Klug, 1986; Segers and Kengen, 1998):

$dB/dt = z*Y*Qmax*(Ac/Ac+Km)*B - D*B$
$dAc/dt = Qmax*(Ac/Ac+Km)*B$

Beide artikelen hebben de D geschat op 0.01 day^{-1} voor anaerobe microorganismen op basis van een review waarin de range 0.004-0.036 day^{-1} was (Pavlosthatis and Gomez, 1991). De originele data van sterfte van anaerobe microorganismen uit de review kwamen van 0.011-0.015 day^{-1} Lawrence and McCarty (1969), 0.036 (Kugelman and Chin (1971) en 0.004 (Wandrey and Aivasidis (1983).

Voor aerobe microorganismen is D geschat op 1 day^{-1} (Segers, 1998) op basis van Nagai et al. (1973) en Sheehan and Johnson (1971). Ook Servais et al. (1985) vond hoge relatieve mortality rates voor aerobe organismen: 0.24-0.79 day^{-1}. Roslev and King (1994) vonden echter 0.21 en 0.11 day^{-1} onder resp. aerobe en anaerobe omstandigheden in C starvation experimenten voor methanotrofen, gebaseerd op activiteitsmetingen. Op basis van biomassametingen (in de vorm van proteinegehaltes) kunnen echter lagere snelheden berekend worden, te weten 0.078 day^{-1} (aeroob) en 0.026 day^{-1} (anaeroob). Op basis van gelabelde biomassametingen komen Roslev en King (1995) ook op lagere schattingen: 0.029 day^{-1} (aeroob) en 0.003 day^{-1} (anaeroob). Het mechanisme van afsterving was ook anders onder aerobe en anaerobe omstandigheden.

Momenteel wordt er gewerkt aan het incorporeren van een temperatuurs-gevoeligheid in deze D. Als eerste benadering wordt een Q_{10} van 2 aangenomen (van Hulzen et al., 1998). Uit de experimenten van Servais et al. (1985) lijkt de Q_{10} echter minder dan 2 te zijn.

De tweede serie artikelen beschouwt alleen een maintenance en geen sterfte snelheid (Esener et al., 1981; Beyeler et al., 1984; Pirt, 1965). In principe is de maintenance op twee manieren te schrijven. De eerste manier is de maintenance te beschouwen als een 'endogeen metabolisme', m, en heeft als eenheid tijd^{-1}. Deze definitie is geintroduceerd door Herbert (1958). De tweede definitie is geintroduceerd door Schulze and Lipe (1964) en wordt uitgedrukt als het extra substraat geconsumeerd per bacterie per tijdseenheid. De twee definities zijn in elkaar over te schrijven met behulp van een yield factor die constant wordt verondersteld en de relatieve groei snelheid, μ (Pirt, 1965; Tros et al., 1996). Doordat de maintenance als deze op de tweede manier is gedefinieerd is gerelateerd aan μ moet heel goed worden gelet op de extrapolatie van dergelijke gegevens naar andere situaties. Op zijn minst zal een correctie voor andere groeiomstandigheden verdisconteerd moeten worden. Nog beter is de maintenance direct om te schrijven naar m. Dat voorkomt problemen.

Doordat beide definities in elkaar zijn om te schrijven is het onjuist om beide methodes in een model te gebruiken, want dan wordt de maintenance overschat. Dit was het geval in de eerste versie van Liesbeths model en bij Leffelaar (1987). Dit is in een latere versie terecht gecorrigeerd.

Op het moment dat de maintenance wordt omgeschreven naar de eerste definitie is m mathematisch niet te onderscheiden van D. Ook in de metingen is het heel lastig deze twee processen te onderscheiden. Aangezien iedereen ofwel D ofwel m gebruikt, zonder voor het andere proces te corrigeren, zijn ook alle metingen eigenlijk de som van D en m. Op grond hiervan lijkt het niet verstandig in een model zowel m als D te introduceren, omdat deze in de literatuur altijd gelumpd zijn en er geen gegevens beschikbaar zijn om ze onafhankelijk van elkaar te schatten. In een latere versie van het model van Liesbeth lijkt het dus verstandig beide parameters te combineren tot één parameter. Door de sterfte op nul te stellen en alleen maintenance mee te nemen is dit in feite ook in het model verwerkt.

Het lijkt alleen mogelijk m en D te onderscheiden door DNA te labelen en het vrijkomen van het label in de tijd te meten. Er wordt n.l. gesteld dat DNA niet aan onderhoud onderhevig is; het vrijkomen van het label is dus een werkelijke sterfte (Servais et al., 1985). In chemostaat experimenten e.d. is zo'n onderscheid niet mogelijk.

b) Varierende sterfte als een functie van de substraat concentratie

Uit de bovenstaande schattingen lijkt het erop dat de relatieve verliessnelheid van de totale biomassa, d', die vanaf nu zal worden gedefinieerd als m+d, een functie is van substraatconcentraties. Dit is ook gevonden door anderen: Esener et al. (1981) vond dat yields daalden met dalende groeisnelheden als een constante maintenance werd ondersteld. De dalende yield met dalende omzettingssnelheden is zelfs al een keer omgeschreven tot een empirische logistische curve (Erickson et al., 1978). Dit effect, doorwerkend tot een niet-lineariteit tussen de specifieke opnamesnelheid en substraatconcentraties (Beyeler et al., 1984; Goma et al., 1979), kan echter heel goed geinterpreteerd worden als een daling in d' met dalende groeisnelheden. Beyeler et al. (1984) vond dan ook hogere maintenance coefficienten bij hogere substraat concentraties (lagere verdunningssnelheden) in een chemostaat. De effecten in zijn studie waren echter vrij gering. Men kan een dergelijk effect interpreteren als een slordiger gebruik van het substraat op het moment dat dat in overvloed aanwezig is en dat organismen zich gaan aanpassen aan lagere substraatconcentraties indien dat nodig is.

Een relatie tussen d' en de groeiomstandigheden kunnen op twee manieren beschreven worden:

De eerste manier is het inbouwen van een afhankelijkheid op een fysiologische manier. Met behulp van de meest eenvoudige versie van Panikov's model is een verandering in de sterfte ingebouwd via de fysiologische constante r_{mo} (Segers, 1998). Op deze wijze daalt de sterfte bij lagere concentraties. Bovendien wordt de verandering in r_{mo} ook beschreven m.b.v. een lag time tau_{mo} om history effecten mee te nemen. Zo'n lag time

is in echte systemen ook gevonden. Een nadeel van deze beschrijving is dat de r_{mo} ook doorwerkt op de Q_{max} en dus ook op de groei waardoor er een kwadratische dubbele monod vergelijking ontstaat voor groei. Het is de vraag of dit is te verantwoorden. Misschien zou het model nog verder vereenvoudigd moeten worden door de r_{mo} niet op de Q_{max} te laten doorwerken.

De tweede methode is het inbouwen van een afhankelijkheid op een kinetische manier (van Bodegom, 1998): Hierbij is uitgegaan van twee kinetische beschrijvingen om de veranderingen in bacteriele biomassa te beschrijven. In de eerste beschrijving wordt gebruik gemaakt van de omzettingssnelheden zoals bepaald m.b.v. de K_M-waarde. in de tweede beschrijving is gebruik gemaakt van de relatieve groeisnelheid, μ, en is de verandering in biomassa dus ondermeer een functie van de K_s-waarde. Door deze beschrijvingen aan elkaar gelijk te stellen ontstaat de volgende vergelijking:

$$d' = \mu_{max} \cdot \frac{\frac{K_s}{K_M} - 1}{1 + \frac{K_s}{[S]}} \qquad (1)$$

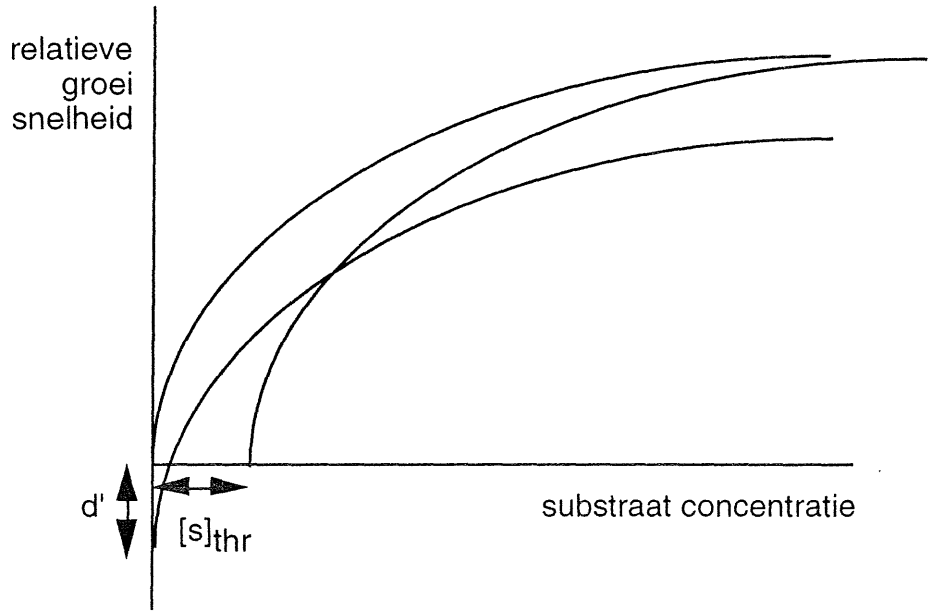
Dit leidt, net als in het geval van Segers (1998) tot soort logistische curve tussen [S], de substraat concentratie, en d'. De helling van deze curve is sterk afhankelijk van de ratio K_s/K_M. Bij deze benadering ontstaat er geen adaptatieperiode, wat niet overeen komt met de werkelijkheid: Roslev en King (1994) vonden na starvation een lag time van 2 uur voordat methaan oxidatie weer op gang kwam en deze periode werd langer (tot 12 uur) naarmate de starvation langer duurde (max. 21 dagen). Wellicht is deze periode in te bouwen door gebruik te maken van Rutter and Nedwell (1994) (**NOG DOEN**). Ook kan nog geprobeerd worden de twee benaderingen in elkaar om te schrijven (**NOG DOEN**).

De sterfteschattingen van verschillende referenties bij verschillende substraat concentraties kan ter validatie en parametrisatie van deze twee beschrijvingen gebruikt worden.

c) Introductie van een threshold concentratie

Een alternatief is dat men verondersteld dat sterfte niet optreedt, maar dat in plaats daarvan er een bepaalde threshold substraatconcentratie is waarboven pas groei optreedt. Daaronder krijgt men dus eigenlijk een negatieve groei, als men de curve consequent zou toepassen. Meestal wordt echter verondersteld dat onder de threshold substraatconcentratie de groei nul is.

Bij deze methode wordt de μ-[S] curve in feite horizontaal verschoven i.p.v. vertikaal verschoven zoals het geval is bij de introductie van een sterfte (zie Figuur 1 voor een curve waarbij constante sterfte is verondersteld). Misschien zijn deze threshold concentraties wel om te schrijven naar de kinetische vergelijkingen m.b.v. de thermodynamische theorie zoals omschreven door Jetten et al. (1990) en Hoh and Cord-Ruwisch (1996) (**NOG DOEN**).



Figuur 1: Vergelijking van verschillende benaderingen om verlies van biomassa te beschrijven

Kinetisch de threshold concentatie, S_{thr}, in een maintenance uitdrukken, is geen probleem en is gelijk aan:

$$S_{thr}=\frac{d'\cdot K_M}{(Q_{max}\cdot Y-d')} \qquad (2)$$

Of als als de redenatie van Tros et al. (1996), die de d' op twee plaatsen in de relatieve groeivergelijking verwerkt, wordt gevolgd:

$$S_{thr}=\frac{d'\cdot K_M}{Q_{max}\cdot Y} \qquad (3)$$